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Kaitlyn M. Dochelli
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**THE INCREASED FREQUENCY OF MICRONUCLEI SEEN IN WOMEN WITH A HISTORY OF
CHILDHOOD SEXUAL ABUSE REFLECTS MORE NUMERICAL THAN STRUCTURAL
ACQUIRED CHROMOSOMAL EVENTS: A DISCORDANT IDENTICAL CO-TWIN STUDY**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

by

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Thursday, December 5th, 2019

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December 2019**

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Abstract

THE INCREASED FREQUENCY OF MICRONUCLEI SEEN IN WOMEN WITH A HISTORY OF CHILDHOOD SEXUAL ABUSE REFLECTS MORE NUMERICAL THAN STRUCTURAL ACQUIRED CHROMOSOMAL EVENTS: A DISCORDANT IDENTICAL CO-TWIN STUDY

Kaitlyn Marie Dochelli, BA

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2019.

**Director: Colleen Jackson-Cook, PhD
Director of Cytogenetics
Department of Pathology**

Childhood sexual abuse (CSA) is a stressful life experience with lasting/far-reaching health and psychopathological consequences. Our laboratory recently identified a significantly increased frequency of acquired chromosomal anomalies (assessed using the cytokinesis-blocked micronucleus assay) in adult female twins exposed to CSA when compared to their unexposed co-twin. The primary aim of this study was to evaluate potential mechanism(s) underlying the observed increases in levels of micronuclei in an expanded group of 90 female identical twins (61 CSA+ females and 29 CSA- females [including a total of 27 MZ co-twin pairs]) using fluorescence *in situ* hybridization (FISH) methodologies, with PNA probes specific for the centromeric and telomeric regions of all chromosomes coupled with the standard CBMN assay, we were able to characterize the chromosomal contents of MN and, thus, gain insight into the mechanisms underlying MN formation. By scoring 100 MN per study participant for the number of centromeric signal(s) and/or telomeric signal(s) present, we categorized the MN as harboring either: (1) terminal fragments (only a telomeric signal); (2) acentric interstitial fragments (no telomeric or centromeric signal); (3) centric interstitial fragments (only a centromeric signal); or (4) an intact chromosome(s) or chromatid(s). We identified elevated

frequencies of intact chromosome-derived MN in CSA+ women as compared to CSA- women ($P=0.014$), implicating chromosome loss as a mechanism potentially underlying the increased frequencies of MN identified in adult females with a history of CSA. MN containing fragmented chromosomes were also observed in all of the study participants evaluated; however MN containing terminal fragments and MN containing acentric interstitial fragments were seen less frequently in CSA+ women compared to CSA- women. This study represents the first time that the chromosomal contents of MN have been evaluated in individuals in the context of a psychosocial factor. As chromosomal loss and breakage contributes to the development of age-related health problems, these observations provide important insight into the biological mechanisms that may underlie the latent morbidity and psychopathology associated with childhood adversity. Future studies aimed at understanding the biological impact of early-life trauma could determine if the observed increase in acquired chromosomal abnormalities results in detectable somatic clonal mosaicism. This knowledge could ultimately be used to develop screening tools to identify individuals “at risk” for negative health outcomes in adulthood.

Introduction

Often cited as one of the most traumatic forms of childhood adversity, childhood sexual abuse (or CSA) is a pervasive problem nationwide and across the globe, affecting as many as one in four girls and one in six boys under the age of 18 in the United States [1,2,10]. Data revealed in the 2016 report on Child Maltreatment in the U.S. indicate that 8.5% of all cases of maltreatment in America's youth fall under the category of sexual abuse, with 57,329 cases of childhood sexual abuse (CSA) being reported that year [3]. One organization, *Darkness Into Light*, asserts that "child sexual abuse is likely the most prevalent health problem with the most serious array of consequences that children face" [1].

The consequences of childhood maltreatment are multi-fold, and include both concurrent and latent effects [4-7]. In addition to the immediate stress experienced at or around the time of a CSA incident, mounting evidence has demonstrated that early-life stress has a significant impact on the lifelong health of the individual [8-11]. In one influential study, investigators observed that exposure to childhood stress predicts vulnerability to premature death in adulthood [12]. Specifically, they observed that individuals who reported experiencing six or more adverse childhood events died, on average, nearly 20 years earlier than those individuals who did not experience childhood adversity. Other investigators have also observed an association between childhood abuse and adult diseases, including (but not limited to) coronary heart disease, cancer, stroke, chronic lung disease, skeletal fractures, liver disease, diabetes, and autoimmune disorders [8-14]. In addition to premature mortality and morbidity, childhood stress has also been associated with an increased prevalence of psychiatric disorders and psychopathologies, including depression, generalized anxiety disorder, panic disorder, phobias, post-traumatic stress disorder, substance abuse disorders, and disruptive behavior [4,6,7,15-19]. As noted by Kiecolt-Glaser et al. (2011), it is clear that "childhood adversities cast a very long shadow" [20].

Although these empirical and epidemiological studies relating exposure to early-life stress with adverse health outcomes in adulthood are provocative, the findings are correlative in nature and do not provide insight into the biological mechanism(s) underlying how a psychosocial factor experienced in childhood can result in disease pathogenesis later in life [21]. In other words, how does exposure to adverse events in childhood “get under the skin” and contribute to the development of disease(s) several decades later?

One possible means for CSA to be biologically “remembered” to cause morbidity and mortality later in life would be if the early-life stressor resulted in DNA-based changes to the somatic genome of an exposed individual [21]. Our research team has proposed that one potential mechanism contributing to the sequelae of health conditions associated with CSA is an increase in somatic chromosomal instability (broadly defined as the loss or gain of intact chromosomes [aneuploidy] or chromosomal fragments) that results from psychological (and possible physiological) stress associated with traumatic early-life experiences. Indeed, using a discordant identical co-twin study design, our research team observed a significantly increased frequency of acquired chromosomal anomalies (assessed using the cytokinesis-blocked micronucleus [CBMN] assay) in adult female twins exposed to CSA when compared to their unexposed co-twin [21]. While the twins exposed to CSA exhibited a 1.63-fold increase in levels of chromosomal instability compared to their unexposed co-twins, it is not known if this increased frequency reflects a generalized increase of all types of somatic chromosomal abnormalities, or if it is differentially associated with structural versus numerical aberrations [21].

Given its efficiency, reliability, and good reproducibility, the cytokinesis-blocked micronucleus (CBMN) assay has become a favored methodology for measuring somatic chromosomal instability [21-25]. A micronucleus is a small, chromatin-containing structure that originates during nuclear division when entire chromosomes or chromosomal fragments fail to migrate properly to their respective spindle poles during mitosis, with this chromatin being excluded from the daughter binucleates following encasement in its own nuclear envelope [26-

27]. Since a micronucleus/micronuclei (MN) are formed as a result of either failure of the chromosome segregation machinery or chromosome breakage, their assessment in cells undergoing division can aid in their recognition [26]. Moreover, to limit the potential for confounding effects due to altered in vitro growth pressures (especially those that may select for or against those cells with an abnormal chromosomal complement), scoring of MN formation is typically accomplished in binucleated cells that have completed one round of nuclear division, but not cytoplasmic division, with the cells being blocked by treatment with cytochalasin-B (Cyt-B), an inhibitor of the microfilament ring assembly required for the completion of cytokinesis. [22-24, 26] Therefore, the CBMN assay provides an accurate estimate of the frequency of MN induction during the first in vitro interphase and mitotic division following culture initiation [21,26].

Because induction of MN has the potential to give rise to daughter cells with altered gene dosage (genomic gains or losses) and because MN formation has been associated with diminished proliferative capacity and aberrant gene expression, increased frequencies of MN could theoretically result in a broad spectrum of adverse outcomes in affected tissues [27]. Indeed, the frequency of MN formation has been shown to increase with advancing age, and elevated MN frequencies have been associated with a variety of chronic age-related health conditions, including (but not limited to) cardiovascular disease, neurodegenerative diseases, and cancer [22,27-32]. Although we have shown that exposure to CSA results in elevated frequencies of MN, a biomarker of chromosomal breakage and/or whole chromosome loss, the primary aim of the current study was to better understand the mechanisms underlying the acquisition of these acquired somatic cell chromosomal changes [21].

As noted above, two basic phenomena leading to MN induction have been described: 1) chromosome breakage; and 2) dysfunction of the chromosome segregation machinery (the mitotic apparatus) [26]. A wide range of genetic and epigenetic mechanisms have been envisaged to underlie these two phenomena [26]. Chromosomal fragments lost in MN likely arise from double- or single-strand DNA breaks, from broken anaphase bridges formed as a

result of complex chromosome rearrangements (such as telomere end fusions leading to formation of a dicentric chromosome or misrepair of two chromosome breaks leading to formation of both a dicentric chromosome and an acentric fragment), or from gene amplification via breakage-fusion-bridge (BFB) cycles when amplified DNA is eliminated through a process known as nuclear budding [26-27]. Whole, intact chromosomes (or chromatids) lost in MN are believed to have their origins in perturbations in kinetochore assembly, spindle fiber misattachment on the kinetochore resulting in aberrant chromosomal orientation, abnormal centrosome number leading to multipolar mitoses, defects in mitotic checkpoint systems, or delays/perturbations in DNA replication [24-26]. Epigenetic alterations to centromeric DNA have also been identified as a mechanism giving rise to MN harboring whole, intact chromosomes [33].

While one cannot distinguish between MN containing intact versus fragmented chromosomes or chromatids using the standard CBMN assay, characterization of the genetic contents of MN can be inferred when the assay is performed in combination with fluorescence *in situ* hybridization (FISH) methodologies [24,26,34]. At the most basic level, using a single centromeric probe, MN arising from chromosome loss (centromere-positive MN) can be distinguished from MN arising from chromosome breakage (centromere-negative MN) by the presence of a centromere, which can be detected by FISH using probes specific for the pericentromeric region of all human chromosomes [24,93]. Most protocols, however, encourage scoring the number of centromeric signals in MN (versus a dichotomous positive/negative assessment) to better understand aneuploid cell production, since MN containing only one centromere may arise through different mechanism(s) than MN containing more than one centromere [93]. More recently, however, the combined use of centromeric and telomeric FISH probes has been proposed to further optimize one's ability to detect exposure-related effects on MN, allowing for the ability to discriminate between aneuploid versus structural errors in MN [34].

While other investigators have begun to study the contents of MN in healthy and disease-state individuals, or in treated cells in culture, the present study is the first to address the contents of MN in individuals affected by a psychosocial stressor. As Norppa and Falck (2003) point out, “understanding the mechanistic origin and contents of MN is essential for the proper use of this cytogenetic end-point in biomarker studies, genotoxicity testing and risk assessment” [24]. Therefore, the primary aim of this study was to evaluate the mechanisms underlying the observed increased levels of chromosomal instability in adult female identical twins exposed to CSA as compared to their unexposed co-twins.

Materials and Methods

2.1 Ethics Statement

Human subjects' research was approved by the Virginia Commonwealth University IRB (protocols #12407 and #179). Written informed consent was obtained from all research participants.

2.2 Sample and Assessment of Childhood Sexual Abuse

Adult female identical twin pairs ascertained for this study were identified from the population-based Virginia Adult Twin Study of Psychiatric and Substance Use Disorders [35] and the Mid-Atlantic Twin Registry (MATR) at Virginia Commonwealth University (VCU). Details of ascertainment are described elsewhere [36-38]. Participants were evaluated for a history of CSA, lifetime diagnoses of psychiatric and substance abuse disorders, family/home environment during childhood, and parental psychopathology [7]. Childhood sexual abuse was assessed through a mailed questionnaire using questions developed by Martin et al. [39]. Adult female identical twins were specifically asked the following:

“Before you were 16, did any adult, or any other person older than yourself, involve you in any unwanted incidents like (i) inviting or requesting you to do something sexual, (ii) kissing or hugging you in a sexual way, (iii) touching or fondling your private parts, (iv) showing their sex organs to you, (v) making you touch them in a sexual way or (vi) attempting or having sexual intercourse?” [36].

Possible answer choices included “never,” “once,” or “more than once.”

To corroborate self-reports of CSA exposure, each participant was asked to answer the same questions about her co-twin's history of CSA [7]. Type of CSA experienced was categorized into one of three classes of hierarchical exposure: 1) no genital contact (sexual invitation, sexual

kissing, or exposing); 2) genital contact without intercourse (fondling sexual touching); or 3) intercourse [7]. Additionally, discordance between twin pairs was classified as either narrow (defined as “one twin reported CSA and the co-twin reported no CSA”) or broad (defined as “one twin reported CSA and her co-twin reported no or a less deviant form of CSA”) [7]. In this study by Kendler et al. [7], the mean age at which participants reported first being exposed to CSA incidents was 10.2 ± 3.5 years. The mean current age of participants included in this follow-up study is 52.0 ± 8.2 years; thus, a latency period of over 40 years has elapsed since the CSA event was initially experienced [36]. Measures of adult psychopathology and substance abuse disorders were assessed in an in-person psychiatric interview as part of the Kendler study [7].

For participation in the current study, following their indication of informed consent, twins who agreed to participate were asked to complete a health history questionnaire and submit a blood specimen (VCU IRB #12407). The blood samples were obtained by a health care provider of the participants' choosing and shipped to our cytogenetics laboratory (priority overnight delivery carrier) at room temperature.

A total of 84 female identical (monozygotic) twins were assessed as part of the current study, including 61 women with a history of sexual abuse in childhood (CSA+) and 23 women who were not exposed to sexual abuse (CSA-). The mean age of CSA+ women at the time of blood draw was 52.68 years (SD 8.25) and the mean age of CSA- women at the time of blood draw was 50.22 years (SD 8.61). Of the 84 female identical twins included in the current study, there were 16 complete twin pairs who were narrowly discordant for CSA exposure (one twin was exposed to CSA [CSA+] and her co-twin was not exposed to any level of CSA [CSA-]), 11 complete twin pairs who were concordant for CSA exposure (both twins were CSA+), and 30 individuals whose co-twin did not provide a specimen. Table 1 illustrates the study design with study participant groups.

Because of the potential for other adverse familial experiences to be shared between co-twins in a CSA discordant twin pair (outside of the exposure to CSA), we also included a reference group to serve as a negative control (n=6 single twins). These twins were selected from a convenience sample of population-based adult twins [22], who had previously been evaluated to determine genetic and environmental factors contributing to micronucleus frequencies. The selection criteria for the control twins was: (1) sex (only females were included since the CSA study cohort was comprised of only females); (2) age (sample included a single twin from female twin pairs evaluated in the previous study who were in the age range of the CSA study participants); and (3) no history of CSA. The average age of these reference individuals was 56.3 years (SD 19.8), which, as expected based on the selection criteria, was not significantly different from the CSA- twin group ($P=0.490$) or the CSA+ twin group ($P=0.673$).

Table 1. Summary of the Twins Ascertained for this Study.

Study Participants	Number of Complete Twin Pairs	CSA Status (n = number of individuals)		Total
		CSA+	CSA-	
CSA Discordant Twins	16	16	16	32
CSA Concordant Twins	11	22	--	22
CSA Group Single Twins	--	23	7	30
Control Group Single Twins	--	--	6	6
Total	27	61	29	90

2.3 Zygosity Assessment

Twin zygosity status was confirmed as described previously [21]. Briefly, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). The patterns of 13 highly polymorphic short tandem repeat sequences (AmpFISTR® Profiler Plus® and Cofiler® kits, Applied Biosystems, Foster City, CA) were compared between co-twins. Twins were classified as monozygotic if the marker data for the co-twins matched at all 13 loci. Co-twins having marker differences were identified as a dizygotic pair.

2.5 Cell Culture

Lymphocytes were isolated from whole blood specimens (to ensure that any erythrocytes that might be present would not confound the recognition and scoring of MN) using Histopaque-1077 (Sigma) and then established in culture according to standard procedures [23]. Forty-four hours after culture initiation, cytochalasin-B was added (3 µg/ml final concentration). Cells were harvested at 72 hours and slides prepared as described previously [22].

2.6 Fluorescence *in situ* Hybridization (FISH) Procedure

The number of centromeric and/or telomeric signals present in MN associated with a binucleated cell was determined by following a dual-color probe strategy, using peptide nucleic acid (PNA) probes localized to: (1) the centromeric regions of all chromosomes (Texas Red); and (2) the telomeres of all chromosomes (FITC). Both probes were purchased from DAKO. PNA probes contain the same nucleotide bases as DNA and RNA, but differ from standard DNA and RNA probes in that the negatively-charged sugar phosphate backbone has been replaced with a neutral polyamide or “peptide” backbone [55]. This synthetic backbone offers PNA probes increased thermal stability and better hybridization properties [55]. The pancentromeric probe used is specific for the α -satellite DNA repeat sequences that are found in all human

centromeres. The pantelomeric probe used is specific for the (TTAGGG)_n repeat sequence found in all human telomeres. The telomeric regions of the chromosomes were visualized following the probe manufacturer's protocol (DAKO). At the completion of the telomere probe hybridization (2 hours at room temperature), visualization of the centromeric regions of the chromosomes was achieved by adding a pancentromeric probe solution to the slide (1 µl of probe to 11 µl of DAKO wash solution for a half slide) and hybridizing for 1 minute at room temperature. The slides were then washed to remove any non-specific hybridization signals and dehydrated (ethanol series). Following air-drying, the binucleates and MN were counterstained with a DAPI solution (DAKO). The probe signals and cells (binucleates and MN) were visualized using a Zeiss epifluorescent microscope equipped with triple and single band pass filter sets. A total of 100 binucleated cells containing at least one MN were scored for each twin.

Using our adaptation of the criteria developed by Lindberg et al. [34], the probe signal patterns were categorized as being consistent with structural (including terminal, centric interstitial, and acentric interstitial fragments) and/or numerical (intact chromosomes/chromatids) aberrations based on the number of signals observed for each probe (Figure 1). The probe signal patterns present in the MN were assessed and classified into the two broad categories of cells showing evidence for structural chromosomal abnormalities (pooled results of MN containing no signal, only pancentromeric signal, or only pantelomeric signal) or numerical abnormalities (including MN containing at least one pancentromeric signal and at least one pantelomeric signal).

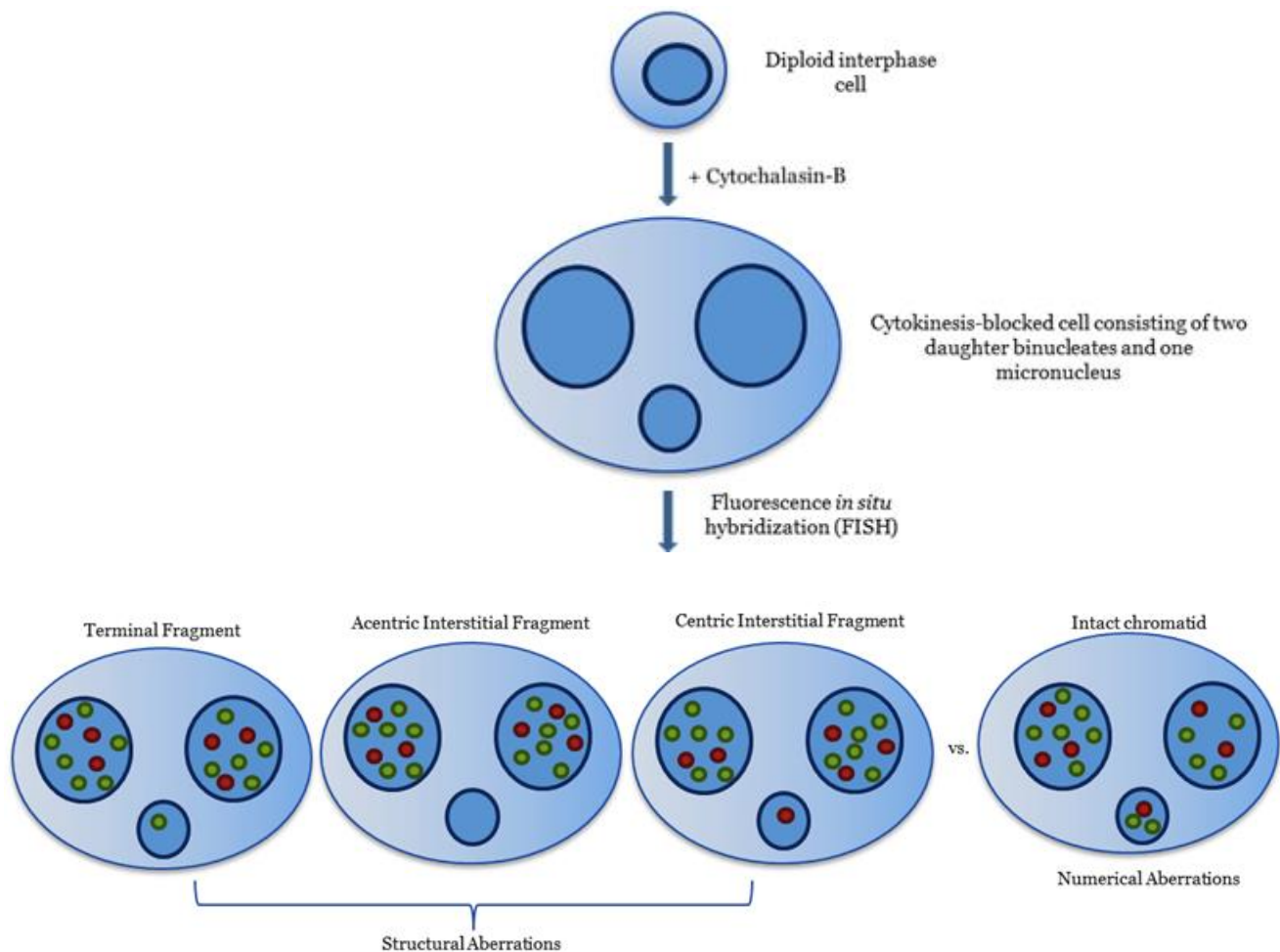


Figure 1. Use of FISH on cytokinesis-blocked binucleates to identify the types of chromosomal events leading to the formation of MN. Pancentromeric (red signal) and pantelomeric (green signal) PNA probes were used to detect the centromeres and telomeres, respectively, of all human chromosomes. One hundred MN were scored for each individual to quantify the frequency of MN arising from structural versus numerical aberrations. Based on the combination of pancentromeric and pantelomeric signals present in the MN, MN were categorized as harboring terminal fragments (only a telomeric signal), acentric interstitial fragments (no telomeric or centromeric signal), centric interstitial fragments (only a centromeric signal), or harboring an intact chromosome(s) or chromatid(s). For simplicity, only three chromosomes are shown in each of the daughter binucleates in the schematic representation above. Also, for simplicity, only one MN is shown per binucleate, though cells can have more than one MN.

2.7 Statistical Analyses

Pairwise comparisons were drawn between discordant co-twin pairs, while group comparisons were drawn between the CSA+ individual twins, CSA- individual twins, and the reference group of individual twins. A variance stabilizing square root transformation was applied to the frequency data for groupwise comparisons, given that MN frequencies have been suggested to follow a Poisson distribution [100]. In addition, given our small sample size, a general influence of exposure to CSA, not differentiated by the type or severity of exposure, was examined in all tests as this was found to be sufficient based on current literature [4,6,7]. The frequency of MN containing intact chromosomes versus chromosomal fragments was compared between the CSA exposed (CSA+) twin group and the CSA unexposed (CSA-) twin group using the nonparametric Wilcoxon rank-sum test. Exact *P*-values were calculated for nonparametric tests. Pairwise comparisons were also performed to evaluate the differences in the frequency of intact chromosome-derived MN versus MN arising through structural errors within twin pairs. A Student's *t*-test was used to assess the difference in the frequency of MN containing intact chromosomes versus chromosomal fragments in the CSA exposed twin as compared to her CSA unexposed co-twin. The Wilcoxon signed rank test was also used as a nonparametric alternative to the paired *t*-test as it could provide additional protection against potential biases with a modest sample size.

Given the possibility for shared familial factors to be present that influence the frequency of acquiring somatic cell chromosomal abnormalities that are not directly related to CSA exposure in a family where abuse was present, the frequency of MN containing intact chromosomes versus chromosomal fragments were compared between the reference twin group as well as the CSA+ and CSA- twin groups. Tests were performed using linear mixed-effect models with Poisson error distribution, accounting for the covariance within families. As described previously [22], two fixed effect terms were included to identify whether an individual belonged to a family where CSA was present:

- A CSA exposure term was coded positive for CSA exposed twins and negative for CSA unexposed and reference sample twins.
- An additional term to indicate belonging to a family where CSA was present was created where twins (both CSA+ and CSA-) identified from the CSA study sample were coded as positive and only reference sample twins were coded as negative.

Evidence for a shared adverse familial effect related to CSA, but beyond that of direct exposure to CSA, would be indicated by a significant coefficient for the second term while controlling for any influence of the first term.

All analyses were performed using the R statistical programming language and a significance value of α (alpha) less than 0.05.

Results

3.1 CSA Exposure is Associated with Increased MN Frequencies: Pairwise and Group Comparisons

As a continuation to our previous findings [22], we evaluated MN frequencies in the expanded group of adult female identical twins who were included in the present study. For discordant twin pairs, identical twins exposed to CSA exhibited, on average, a 1.53-fold increase in the occurrence of MN compared to their unexposed co-twin (Appendix, Section 1). The mean [SD] MN frequency in CSA+ twins from the discordant twin pairs was 22.0 [11.5] per 1000 binucleated cells compared to 14.4 [5.4] in their CSA- co-twins. The absolute MN frequency values were greater in the CSA+ twins for 12 out of the 16 discordant twin pairs evaluated in the present study (Figure 2). A paired t -test comparing MN frequencies within discordant twin pairs indicated a significantly higher frequency of MN formation in the CSA+ twins ($t_{15}=2.83$, $P=0.013$) and a significant Wilcoxon signed rank test was also observed ($W=522$, $P=0.021$). In contrast to the results from the discordant twin pairs analysis, the MN frequencies in CSA concordant co-twins was not found to be significantly different (Paired t -test [$t_3=-0.611$, $P=0.584$]; Wilcoxon signed rank [$P=0.625$]) and showed MN frequencies that aligned with the values seen in the CSA+ twins from the discordant pairs (Figure 2).

Group comparisons also showed that female twins exposed to CSA ($n=61$) have a significantly higher frequency of MN compared to unexposed twins ($n=23$) (Welch Two Sample t -test; $P=0.015$) (Figure 3). The overall mean [SD] MN frequency in CSA+ twins was 22.5 [9.7] per 1000 binucleated cells compared to 16.5 [8.2] in CSA- twins. Based on results from univariate linear mixed-effects modeling, CSA exposure was identified as a significant factor associated with MN frequency ($P=0.025$). In the multivariate model analysis, CSA exposure remained significant ($P=0.007$), and an additional effect attributable to the shared family

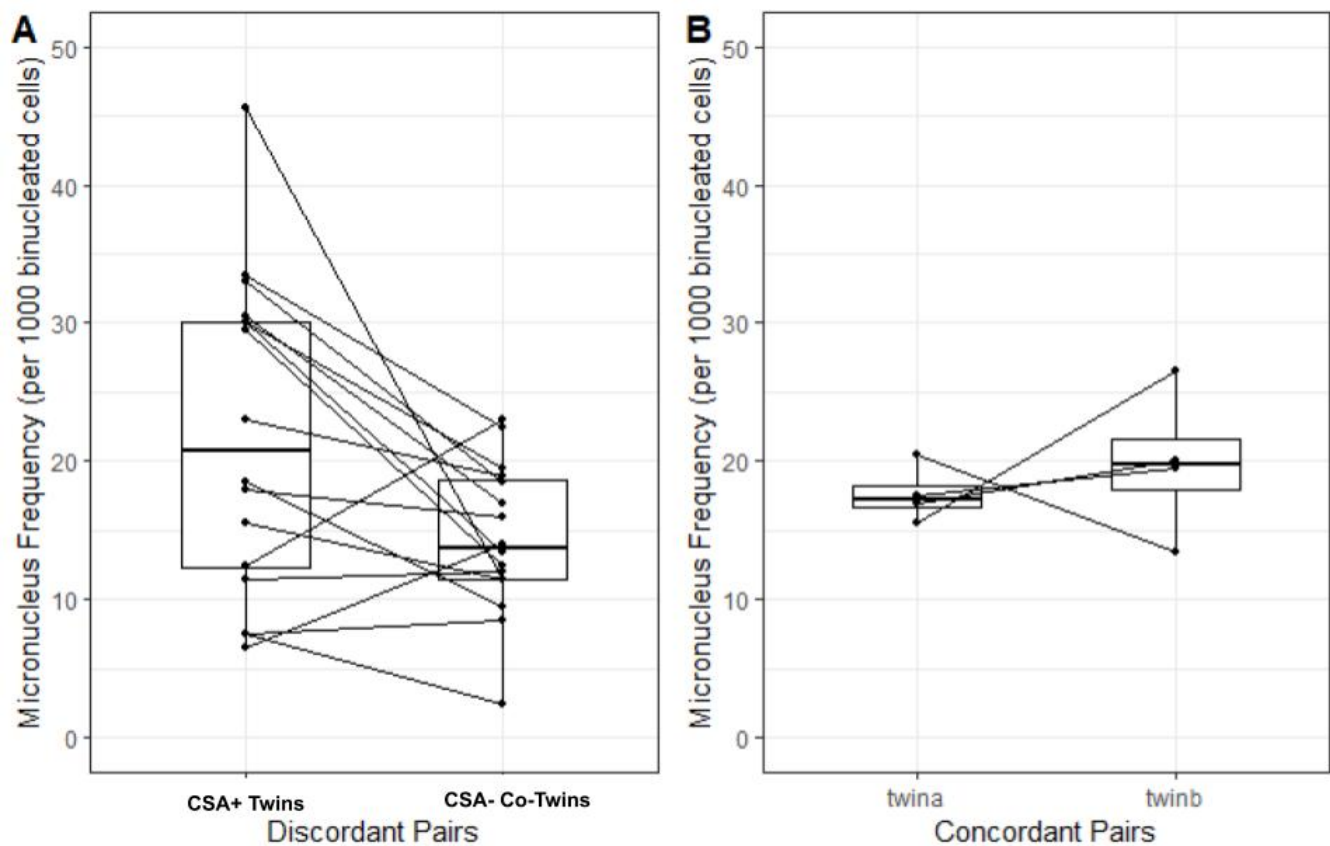


Figure 2. Pairwise comparisons of MN frequencies in adult identical female twin pairs who are (A) discordant ($t_{15}=2.83$, $P=0.013$) or (B) concordant ($t_3=-0.611$, $P=0.584$) (Part B) for exposure to CSA. For the discordant twin pairs, the identical twins exposed to CSA exhibited, on average, a 1.53-fold increase in the occurrence of MN compared to their unexposed co-twin. MN frequencies in CSA concordant co-twins was not found to be significantly different.

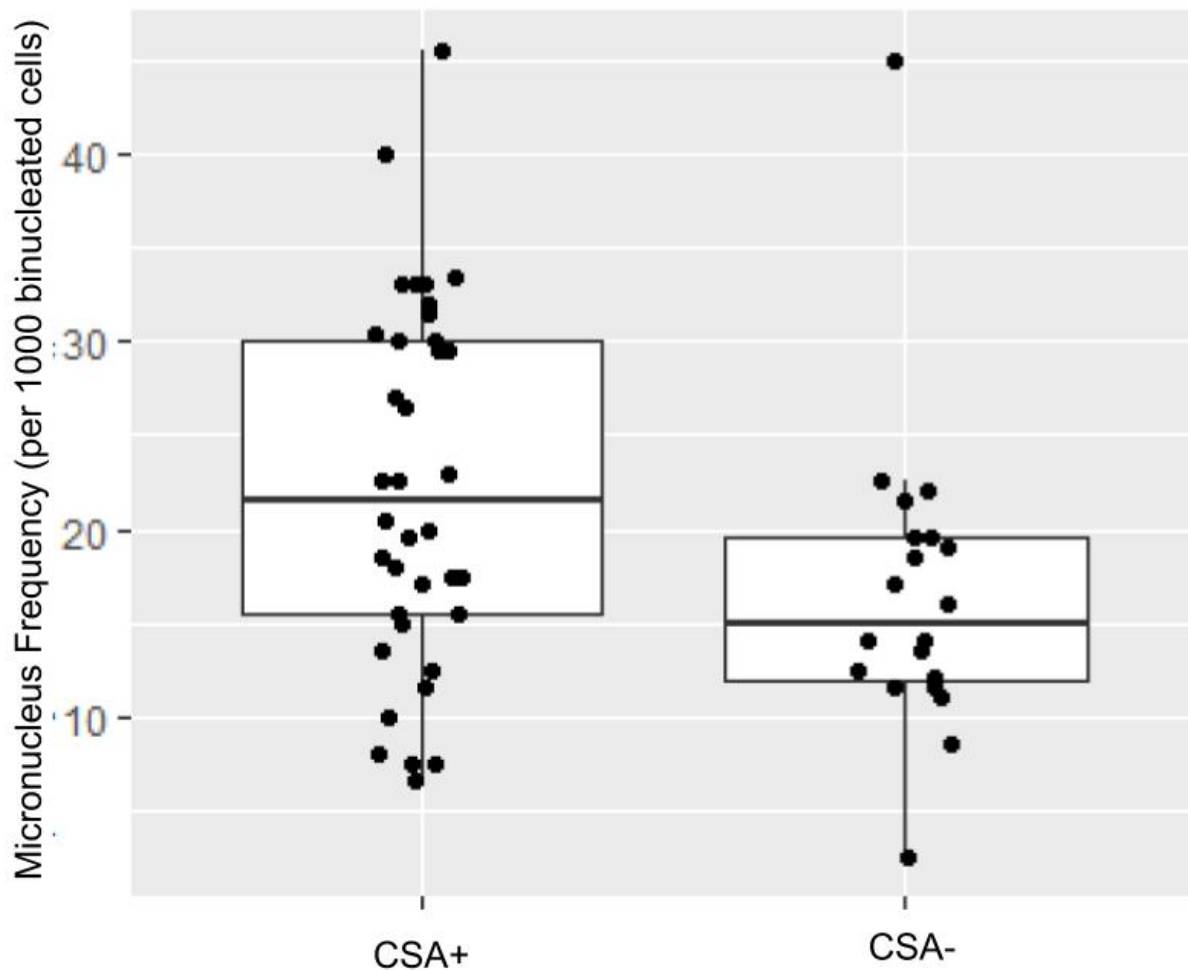


Figure 3. Group Comparison of MN frequencies between the CSA-exposed and CSA-unexposed groups.

The box and whiskers plot shows the distribution of MN frequencies in the CSA+ and CSA- individual twins. The median frequency is shown as a horizontal line within the box. The overall mean [SD] MN frequency in CSA+ twins was 22.5 [9.7] per 1000 binucleated cells compared to 16.5 [8.2] in CSA- twins. Using a Welch Two Sample t-test, the difference in MN frequency between CSA exposure groups was found to be significant ($P=0.015$). Based on results from linear mixed-effect models, CSA exposure status was identified as a significant factor associated with MN ($P=0.025$).

environment was found to be approaching significance ($P=0.056$). In accordance with the findings of our previous study, age was not significant in predicting overall MN frequency using linear mixed-effect models ($P=0.199$).

3.2 CSA Exposure is associated with an increased frequency of MN containing an apparently intact chromosome or chromatid in group comparisons, but not pairwise tests

The frequency of MN observed to harbor intact chromosomes (or a single chromatid) as compared to the frequency of MN observed to contain chromosomal fragments (including, terminal, centric interstitial, and acentric interstitial fragments), based on exposure to CSA, is illustrated in Figure 4 (Appendix, Section 2). For all CSA+ and CSA- twins evaluated in this study, the majority of MN scored contained at least one centromere signal and at least one telomere signal, suggesting that intact chromosome(s) or chromatid(s) had been excluded into the MN. The overall mean frequency of MN containing an intact chromatid or chromosome(s) was 63.6 [20.3].

Given that age is a known predictor of MN frequency and has been shown to influence the frequency of intact chromosome-derived MN in some studies [24], it was important to determine if there was an age difference between CSA+ twins and CSA- twins as the complete dataset includes a subset of twins who were singletons and did not have a participating co-twin of matched age. Indeed, there was no significant age difference between the two groups ($P=0.243$). Using a Wilcoxon rank sum test, we determined that women with a history of CSA exposure displayed significantly elevated frequencies of intact chromosome-derived MN (mean of 67.0 [19.7]) as compared to women without a history of abuse (mean of 54.7 [19.5]) (Figures 4 and 5) ($W=456.5$, $P=0.014$). By contrast, the mean count of intact chromosome-derived MN for the reference twins (negative controls) studied was 40.8 [19.3]. The frequency of intact chromosome-derived MN observed in the reference twins was significantly lower than the

frequency in CSA+ twins ($W=303$, $P=0.009$), but was not significantly different from the frequency in CSA- twins ($W=98$, $P=0.125$). Using linear mixed-effects modeling, CSA exposure was significant in predicting the frequency of MN containing an intact chromatid or intact chromosome ($P=0.011$). An adverse familial effect shared by twins identified from families where CSA was present was also found to be significant in predicting the frequency of MN arising from an intact chromosome or chromatid ($P=0.004$). Shared familial experiences remained significant above and beyond the effect of CSA in our multivariate model when both CSA exposure and familial environment were regressed on the frequency of whole chromosome-derived MN ($P=0.025$), while the effect of CSA was only found to be approaching significance ($P=0.073$). Of note, CSA exposure and shared familial environment were highly correlated ($\rho=0.975$), which suggests that the effect of CSA exposure could not be fully disentangled from the potential effect of an adverse familial effect in our model.

Unlike the results from our group comparisons, the results from pairwise tests performed on CSA discordant twin pairs did not indicate a significant difference in the frequency of intact chromosome-derived MN between the CSA+ and CSA- co-twins. A paired t -test comparing frequencies of MN containing an intact chromosome or chromatid in CSA+ twins (mean of 49.0 [SD 14.8] as compared to her discordant CSA- co-twin (49.46 [16.9]) did not show significance ($t_{15} = -0.086$, $P=0.933$) (Figure 6). Similarly, results from the nonparametric Wilcoxon signed rank test were also not significant ($P=0.940$). As a positive control, we also compared the frequencies of intact chromosome-derived MN between co-twins from the CSA concordant twin pairs (Figure 6). Pairwise tests performed on CSA concordant twin pairs did not show evidence for a significant difference, as indicated by a paired t -test ($t_{10} = -1.402$, $P=0.191$) and the Wilcoxon signed rank test ($P=0.213$). Of note, the overall mean frequency of MN arising from numerical events in the CSA concordant twin group was 76.4 [17.9], which is higher than the overall group mean of 63.6 [20.3].

MN arising from structural anomalies were also observed (overall mean MN frequency of 36.4 [SD 20.3]), and included MN harboring terminal fragments (mean of 27.5 [17.3] CSA+; 37.1 [16.5] CSA-), centric interstitial fragments (1.69 [1.4] CSA+; 2.4 [2.4] CSA-), as well as acentric interstitial fragments (3.8 [4.1] CSA+; 5.8 [4.9] CSA-). Overall, the difference in the frequency of MN resulting from structural chromosomal errors was significant between the CSA exposed and CSA unexposed groups, as assessed using a Wilcoxon rank sum test ($W=426.5$, $P=0.014$). Significant differences were also observed between structural anomaly subcategories, with twins having a history of CSA showing a significantly smaller frequency of MN containing terminal fragments ($W=481.5$, $P=0.028$) and acentric interstitial fragments ($W=495$, $P=0.037$).

In addition to comparing means between twins based on CSA exposure, we also evaluated the potential for confounding factors to influence the frequency of MN containing intact chromosomes versus fragmented chromosomes. Using linear mixed-effects modeling, age was not significant in predicting the frequency of intact chromosome-derived MN ($P=0.720$). Similarly, overall MN frequency was also not significant in predicting the frequency of MN arising from a numerical event ($P=0.519$). To determine if the “severity” of CSA exposure was associated with the frequency of MN arising from a numerical aberration, we compared values in twins based on their CSA experience (for example, non-genital exposure; genital exposure; or intercourse). Using general tests of association, we did not observe a significant association between CSA class and the frequency of MN arising from a numerical event ($P=0.472$).

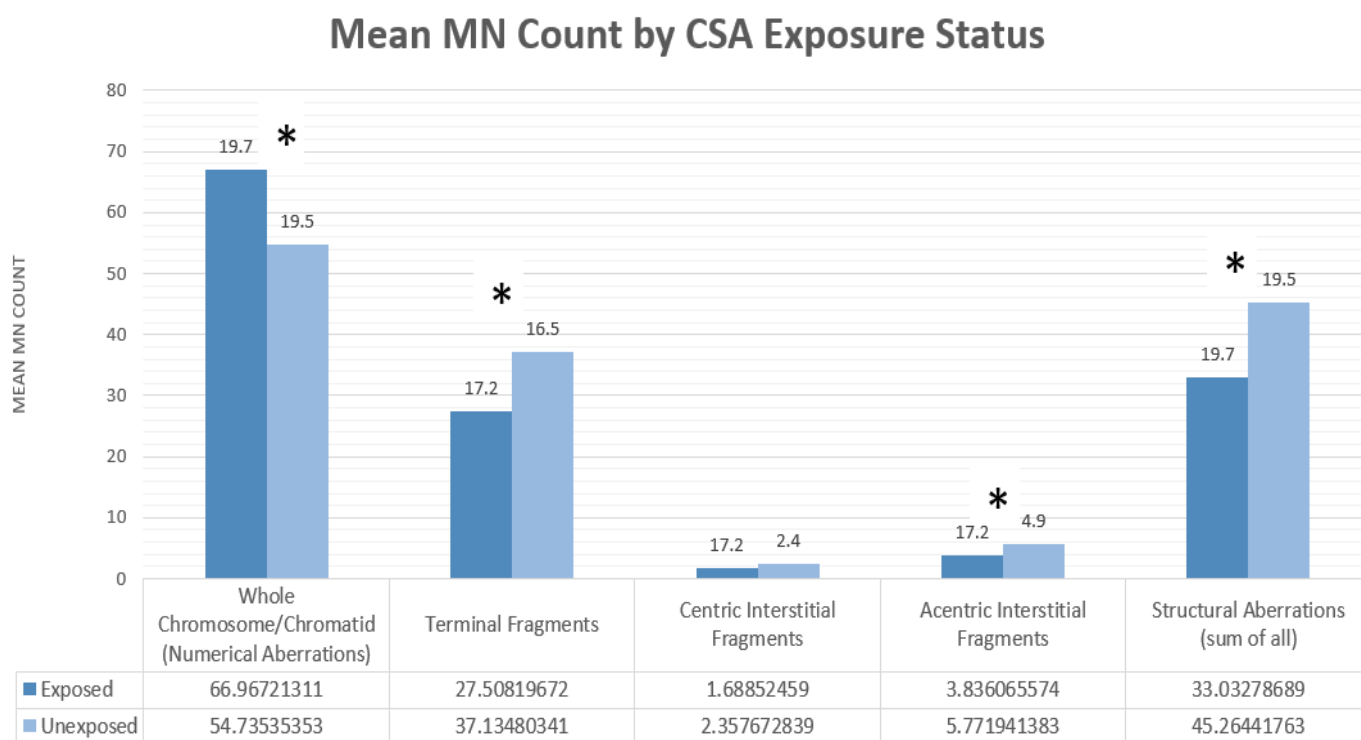


Figure 4. Frequency of MN observed to harbor intact chromosomes (or a single chromatid) as compared to the frequency of MN observed to contain chromosomal fragments (including, terminal, centric interstitial, and acentric interstitial fragments) in Adult Female Identical Twins. For each individual, 100 MN were scored. Means are shown in the table and standard deviations are listed above each individual bar. Asterisks indicate significant differences between the CSA exposed (CSA+) and CSA unexposed (CSA-) twin groups, where a single asterisk (*) indicates a p-value of less than 0.05 and a double asterisk (**) indicates a p-value of less than 0.01.

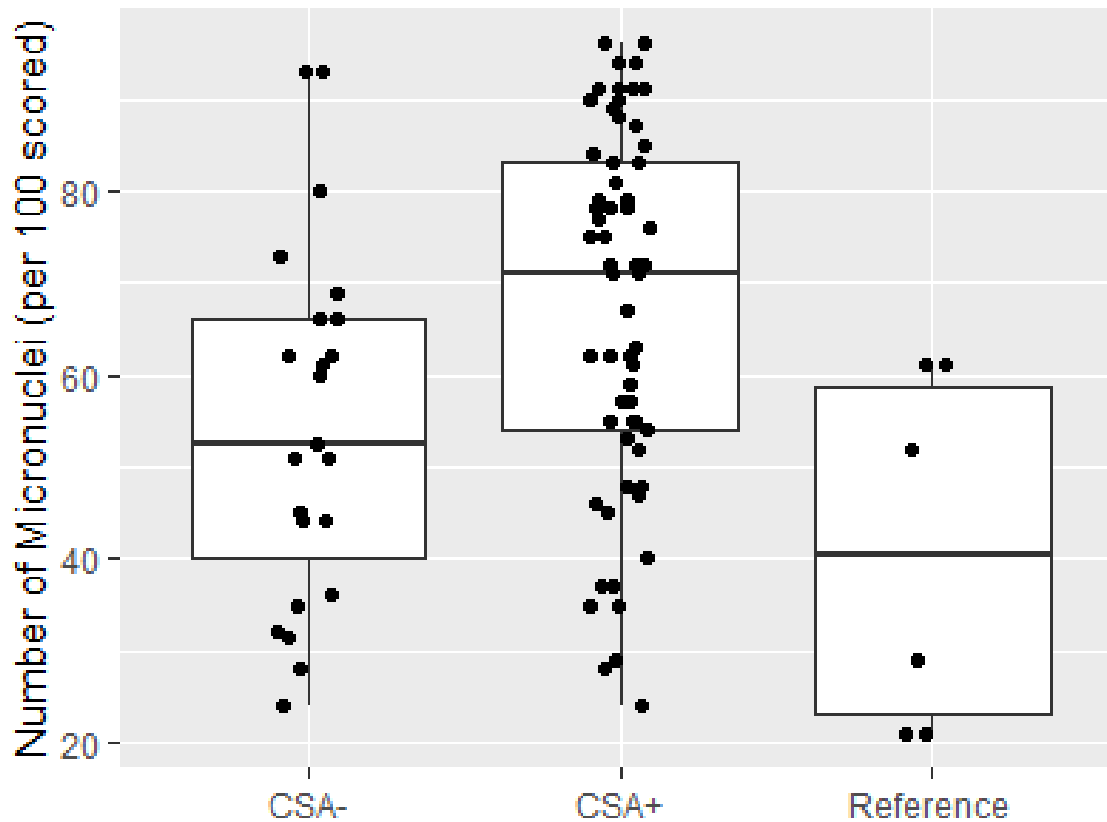


Figure 5. Number of MN containing an apparently intact chromosome/chromatid by exposure category. The mean frequency of intact chromosome-derived MN was significantly higher in adult female identical twins with a history of CSA (CSA+; mean [SD] 67.0 [19.8]) as compared to unexposed adult female identical twins (CSA-; 54.7 [19.5]) ($P=0.014$). The mean frequency of whole chromosome/chromatid-derived MN for the reference twins (negative controls) was 40.8 [19.3], which was significantly lower than the CSA+ twin group ($P=0.009$), but not the CSA- twin group ($P=0.125$).

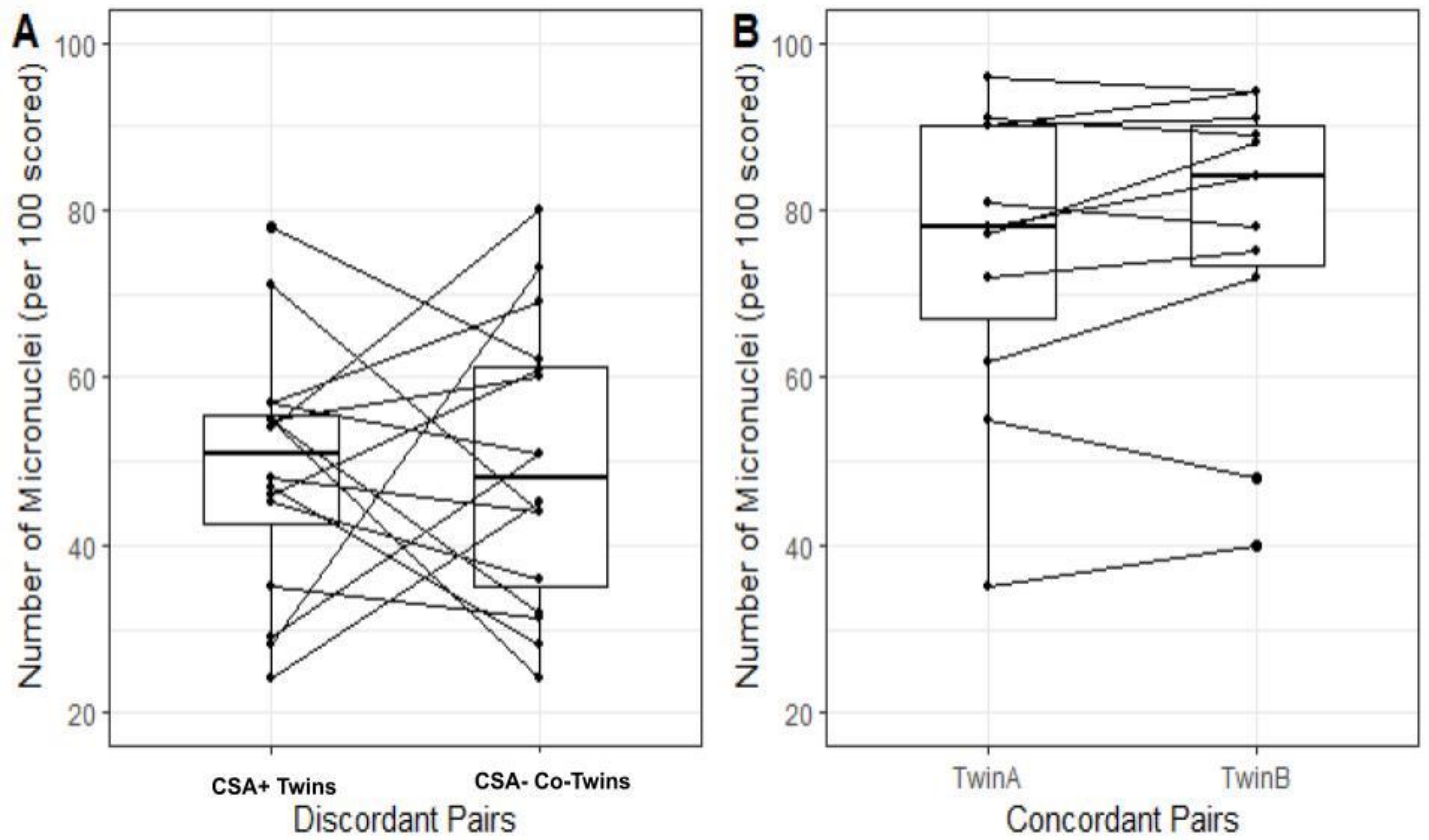


Figure 6. Pairwise comparisons of frequencies of MN deriving from an apparently intact chromosome(s) or chromatid(s) in adult identical female twin pairs who are (A) discordant ($t_{15}=-0.086$, $P=0.933$) or (B) concordant ($t_{10}=-1.402$, $P=0.191$) for exposure to CSA. In nine out of the 16 discordant twin pairs, the frequency of MN arising from a numerical event was higher in the CSA+ twin than her CSA- co-twin.

Discussion

The aim of this study was to shed light on the mechanism(s) responsible for the elevated MN frequencies in adult female identical twins with a history of CSA as compared to adult female identical twins who were not exposed to CSA. The present study is the first of its kind to evaluate the contents of MN in the context of exposure to a psychosocial factor. There were several main findings and observations:

- The present study, which included an expanded group of study participants, was in agreement with the previous finding by our laboratory that MN frequencies are elevated in women with a history of CSA exposure. MN formation was again shown to be significantly increased in CSA exposed twins as compared to their CSA unexposed co-twins. No significant difference was found in MN frequencies between CSA concordant co-twins, providing further support that CSA exposure (rather than other environmental influence(s)) is the true driver of the observed variance between co-twins.
- Although the association of advancing age and increased MN frequency is well-established, this result was not identified here, likely due to the fact that the twins selected for this study were from a narrow age range [22].
- Using FISH methodologies, coupled with the standard CBMN assay, it was possible to characterize the contents of MN based on the presence or absence of centromeric and/or telomeric signals. While studies of this kind have been performed in healthy individuals and individuals in an active disease state (i.e., Alzheimer's disease [27]) as well as on cells that have been treated *in vitro*, to our knowledge, the study presented here represents the first time that this combined technique has been used to evaluate the contents of MN in relationship to a psychosocial factor.

- Our data revealed that CSA exposure was significant in predicting the frequency of MN containing an apparently intact chromosome or chromatid. The rate of MN induction arising from a numerical event was independent of the influence of age or overall MN frequency; thus, neither potential confounding factor was required to remain in the final multivariate model. However, because CSA exposure and familial environment were so highly correlated in the present study, it was impossible to tease apart the shared experience of growing up in a family where abuse was present from the effect of CSA. It remains an interesting question to explore whether it is the impact of the shared adverse familial environment or direct exposure of CSA that may be influencing the frequency of intact chromosome-derived MN.
- Pairwise analysis of the CSA concordant twin pairs illustrates the concordance of elevated intact chromosome-derived MN frequencies in identical twins with similar exposure to CSA. Given the lower than average frequencies of MN containing an apparently intact chromosome/chromatid seen in both the CSA+ and CSA- discordant twins, one might conjecture that there may be shared genetic factors that play a role in MN formation, particularly in the formation of MN arising from a numerical event.
- MN arising from structural abnormalities were also observed in twins with and without a history of sexual abuse in childhood; however, MN harboring terminal fragments and acentric interstitial fragments were seen less frequently in CSA+ twins than in CSA- twins. It is possible that these observations reflect true smaller frequencies of structural errors resulting in the formation of MN in CSA+ twins compared to CSA- twins (for reasons that likely cannot be gleaned from our data alone), and that CSA exposure is associated with differential mechanism(s) giving rise to MN. Alternatively, one could conjecture that because this study was

limited to 100 observations per individual, the ratio of MN arising from structural abnormalities in CSA+ twins would be smaller simply due to the fact that the frequency of intact chromosome-derived MN in CSA+ twins was higher.

- Additional studies to answer mechanistic questions of the biological or cellular impact of psychological stress in childhood are needed to better understand the increased chromosomal loss and breakage in female identical twins exposed to CSA.

Since aneuploidy and chromosomal instability contribute to the acquisition of age-related diseases, these observations are an important step toward unraveling the biological impact of CSA on adult health. Our data suggest that CSA exposure is associated with an increased rate of MN induction arising primarily from intact chromosome/chromatid loss, which would likely result in a somatic imbalance in one or both of the daughter binucleates. One could speculate that it is this somatic imbalance which may then be a contributory factor to the latent disease pathogenesis observed in individuals exposed to adverse early-life events. In addition, the observed differences in rates of MN containing terminal and acentric interstitial fragments in CSA+ versus CSA- women suggests that the mechanisms resulting in MN formation may differ based on the experience of childhood trauma.

4.1 A Study Aimed at Contributing to a Growing Body of Knowledge Identifying Cellular and Chromosomal Events Underlying the Formation of MN

Given that an intact chromosome/chromatid will contain a centromere associated with a kinetochore structure, investigators have used centromere-specific DNA sequences or centromeric (kinetochore) proteins to infer the presence of an intact chromosome/chromatid in MN [24]. Early investigators used what is known as the CREST method to study the contents of MN, whereby anti-kinetochore antibodies derived from the serum of patients with scleroderma CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and

Telangiectasia) detect the presence of the kinetochore structure of the centromeric region. However, a more stringent (and accurate) description of an intact chromosome or chromatid requires the recognition of sequences from one tip of the chromosome, through the centromere, to the other tip of the chromosome. In the current study, we applied FISH methodologies using probes (1) specific for the α -satellite DNA that is found in all human centromeres and (2) specific for the (TTAGGG)_n repeat sequence found in all human telomeres. Findings from previous studies evaluating the average percentage of MN containing intact chromosomes (or chromatids) in induced or spontaneously-occurring MN using either the CREST antibody assay or FISH methodologies are summarized in Tables 2-4.

The results of these previous studies highlight several significant findings that can be deduced from the combined data that are relevant to the current study. The majority of MN (either arising spontaneously or in response to an exposure) contain chromatin from a single chromosome/chromatid, rather than a chromosomal fragment. In the previous reports, estimates of intact chromosomes in spontaneously-occurring MN in cultured human lymphocytes ranged from approximately 30% to 80% (Table 2). Additionally, women were found to have higher frequencies of centromere-containing MN than men [92]. In the present study, nearly 64% of MN in cultured lymphocytes were centromere-positive (C+) MN, which aligns nicely in the wide range of intact chromosome-containing MN frequencies observed elsewhere [24].

Advancing age is associated with an increase in both the prevalence and frequency of kinetochore-positive (K+) MN [83-85] and C+ MN [86-90] in human lymphocytes. Thierens et al. [90] calculated that 80% of the effect of age on overall MN frequencies is due to C+ MN. As will be discussed in greater detail in a later section, individuals exposed to significant early-life stress have been conjectured to develop a “premature aging phenotype,” resulting in increased morbidity and mortality in adulthood. Our observation that CSA+ women have elevated frequencies of C+ MN (evidenced by the elevated frequency of intact chromosome-derived MN)

are consistent with the hypothesis that these women have an increased “biological” age compared to their “chronological” age.

The age-related increase of C+ MN has been shown to primarily reflect an age-dependent micronucleation of the sex chromosomes [24,92]. In particular, the X chromosome has a tendency to lag behind in anaphase, being more frequently micronucleated than autosomes in females [22,24]. Interestingly, preliminary Spectral Karyotyping (SKY) analysis from our laboratory on the same population of CSA+ and CSA- twins studied here showed that MN contained chromatin from chromosomes X (23%; $\chi^2_{23}=7122$; $P<0.001$) and 8 (8%; $\chi^2_{23}=31.51$; $P<0.001$) were seen more frequently than expected by chance [60].

Analyzing contents of MN has also been used for genotoxicity testing or for biomonitoring of genotoxic exposure to assess possible clastogenic or aneugenic effects of various disease states, treatment of cells *in vitro*, or exposure to a potentially hazardous substance (reviewed in Tables 3 and 4) [24]. Upon reviewing the results from the diverse studies in this field, it is notable that a wide range of exposures (therapeutic, occupational, environmental, or social [as evaluated in this study] are associated with increased MN frequencies, and the majority of these MN likely arise from malsegregation/loss of an intact chromosome or chromatid.

Of the structural abnormalities assessed in our study, we saw the highest frequency of MN containing terminal fragments, with MN containing acentric or centric fragments being less frequently observed. Interestingly, using pancentromeric and pantelomeric DNA probes, Lindberg et al. (2007) evaluated the contents of MN as well as nuclear buds, and these investigators observed that nuclear buds preferentially harbored terminal fragments and acentric interstitial fragments [90]. Nuclear budding has been proposed as a defense mechanism of the cell - a means to remove extra chromosomal double minute that formed from gene amplification via BFB cycles when amplified DNA is selectively localized to sites at the periphery of the nuclear and eliminated [26]. Based on our observation that CSA+ twins have lower frequencies

of MN containing terminal fragments and acentric interstitial fragments, one could speculate that exposure to CSA may result in cells losing the ability to remove additional genomic material through the process of nuclear budding, which could cause adverse outcomes on the affected cells.

Although one may be inclined to speculate about specific mechanism(s) leading to the micronucleation of intact chromosomes or chromatids versus fragmented chromosomes, data obtained from the CBMN assay with FISH using pancentromeric and pantelomeric probes is insufficient to tell the complete micronucleation story. According to Norppa and Falck, the “two basic phenomena leading to the formation of MN in mitotic cells are chromosome breakage and dysfunction of the mitotic apparatus” [24]. Beyond these two simplistic mechanisms, MN may also arise from fragments derived from broken anaphase bridges formed as a result of chromosomal rearrangements, such as dicentric chromatids or ring chromosomes [24,92]. Centromere-positive MN are generally thought to result from mitotic spindle dysfunction, but MN containing a single centromere may arise through different mechanisms than MN containing more than one centromere [92]. As described by Iarmarcovai et al. [92], MN containing a single centromere may be the result of impaired chromosome migration, whereas MN containing two centromeres may be the result of an intact duplicated chromosome with a centromere defect (or a broken dicentric chromosome). Alternatively, MN with three or more centromere signals may be caused by mitotic disruption events, such as centrosome amplification, or replication *in vivo* (MN retained and replicating). Thus, while the results of the current study provide some insights into the mechanisms leading to increased micronucleation in women with a history of psychological stress in childhood, it would not be appropriate to suggest that malsegregation/mitotic spindle defects alone are responsible for the increased frequency of MN seen in CSA+ twins.

Finally, while the mechanisms underlying MN formation are well delineated, the potential post-mitotic fate of MN after their formation in the micronucleated cell is not yet fully understood

[26]. it should be mentioned that while it was once assumed that after MN form, their chromatin contents were eliminated from cells (potentially resulting in either a somatic imbalance in the remaining binucleates or a “correction” of an imbalance), it is now known that some MN are retained in cells and that their chromatin contents can be reincorporated into a cell [92]. Thus, three outcomes for the post-mitotic fate of MN include: (1) expulsion from the cell; (2) reincorporation into the main nucleus; (3) retention within the cell’s cytoplasm as an extra-nuclear entity [92].

Table 2. Summary of Previous Reports Denoting Presence of Centromeric and/or Telomeric Chromatin in Spontaneously-Occurring MN in Healthy Human Subjects.

Citation	Tissue	Study Sample	Technique	Key Findings
Fenech et al. (1989) [83]	Human Lymphocytes	Young (20-35 years) and elderly (>65 years) subjects	Detection of centromeres using anti-kinetochore antibodies	Results for spontaneously occurring micronuclei in young and elderly indicated that 42(\pm 6)% and 50(\pm 6)%, respectively, contained kinetochores
Norppa et al. (1993) [84]	Human Lymphocytes	Healthy male subjects	Immunofluorescence using antikinetochore (CREST) serum and by a centromeric alphoid DNA oligomer probe (<i>in situ</i> hybridization, ISH)	The binucleate lymphocytes had high prevalence of K+MN (mean 79-84%) In the ISH analysis, the majority of MN in binucleated cells were positively stained (mean 58-62%)
Catalan et al. (1995) [85]	Human Lymphocytes	Six females < 30 years Six females > 50 years	Fluorescence <i>in situ</i> hybridization with a probe for centromeric alphoid consensus sequences and a X-specific centromeric probe to detect the presence of any chromosomes and the X chromosome, respectively	Presence of C+ MN was significantly higher among the older females (51.5%) than the younger females (34.4%) Percentages of C+ MN with one, two, and three or more signals were, respectively, 20.5%, 10.3%, and 3.5% for the younger group and 29.1%, 16.3%, and 3.6% for the older group
Scarpato et al. (1996) [86]	Human Lymphocytes	20 subjects (10 males and 10 females)	Fluorescence <i>in situ</i> hybridization using dual-color hybridization with pancentromeric and acrocentric-specific DNA probes	MN were found to contain acrocentric chromosome(s) at an average frequency of 26.8%, as compared to a 60% frequency of centromere-positive MN The percentage of total C + MN increased significantly with advancing of subjects ($r = 0.695$, $p < 0.001$, while no relationship was found between the frequency of micronuclei containing acrocentric chromosome(s) and donor age.
Surrallés et al. (1996) [87]	Human Lymphocytes	Female subjects	Fluorescence <i>in situ</i> hybridization with pancentromeric DNA probes or probes specific for the X chromosome	Micronuclei mostly contained intact chromosomes (71.6%), especially the X chromosome (28.5%) Cell culture (72 h) enhanced the frequency of micronuclei harboring acentric fragments 2.9 fold and the X chromosome 1.5 fold
Catalan et al. (1998) [88]	Human Lymphocytes	Male subjects	Fluorescence <i>in situ</i> hybridization using pancentromeric DNA probes	>50% of the centromere-containing MN had a single centromere signal, ~25% contained two signals, and ~10% contained three or more signals

Leach and Jackson-Cook (2001) [89]	Human Lymphocytes	Three females (aged 28, 42, and 72)	Fluorescence <i>in situ</i> hybridization and SKY	<p>Most of the MN of three women (82% in 28-year-old, 69% in 42-year-old, and 80% in 72-year-old) had one centromeric signal. The percentages of MN with more than 2 signals were 5%, 12%, and 9% in the 28-, 42-, and 72-year-old, respectively</p> <p>The SKY studies also showed that the majority of MN were comprised of chromatin from a single chromosome</p>
Lindberg et al. (2007) [90]	Normal and folate-deprived 9-day cultures of human lymphocytes	N/A	<p>Fluorescence in situ hybridization with pancentromeric and pantelomeric DNA probes</p> <p>(characterized the contents of 894 nuclear buds and 1392 micronuclei)</p>	<p>Interstitial DNA was more frequently found in nuclear buds (43%) than in MN (13%) DNA with only telomere label or with both centromere and telomere label was more prevalent in MN (62% and 22%, respectively) than in nuclear buds (44% and 10%, respectively)</p> <p>Folate deprivation increased the frequency of nuclear buds and MN harboring telomeric DNA and nuclear buds harboring interstitial DNA</p> <p>Model is proposed that MN in binucleate lymphocytes primarily derive from lagging chromosomes and terminal acentric fragments during mitosis, while nuclear buds are suggested to originate from interstitial or terminal acentric fragments</p>
Lindberg et al. (2008) [34]	Human Lymphocytes	Unexposed, healthy subjects (two men and two women) 35–56 years of age	<p>Fluorescence <i>in situ</i> hybridization using directly labelled pancentromeric and pantelomeric DNA probes</p> <p>(50 MN scored per individual)</p>	<p>Majority of C+ MN contained telomeric sequences (T+): Most of the C+ T+ MN had one centromere and two or one telomere signals, suggesting that single chromatids were more frequently present in MN than both sister chromatids</p> <p>Among the C– MN, telomere signals were found in the majority, showing that fragments in MN were mostly terminal</p> <p>Most C– T+ MN had one telomere signal, indicating higher prevalence for chromatid-type than chromosome-type terminal fragments</p>

Key: Micronuclei (MN) containing centromere signal (C+ MN) or telomere signal (T+ MN); Spectral Karyotyping (SKY).

Table 3. Summary of Previous Reports Denoting the Presence of Centromeric and/or Telomeric Chromatin in Spontaneously-Occurring MN in Patients with an Active Disease.

Citation	Tissue	Study Sample	Technique	Key Findings
Migliore et al. (1999) [91]	Human Lymphocytes	Systemic lupus erythematosus (SLE) and systemic sclerosis (SS) patients	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	<p>As compared with controls, SS patients (but not SLE patients) displayed significantly higher MN frequencies</p> <p>Data showed a significant prevalence of centromere-negative MN in SLE and SS patients with ACA–/Scl70+ antibody status</p> <p>Data also showed a significant prevalence of C+ MN in SS patients with ACA+/Scl70– status</p>
Baciuchka-Palmaro et al. (2002) [92]	Human Lymphocytes	Untreated cancer patients and controls	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	Untreated cancer patients displayed a two-fold higher average frequency of centromere-negative MN compared to controls
Migliore et al. (2011) (Review) [93]	Human Lymphocytes	Alzheimer's disease and Parkinson's disease patients	<p>Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe</p> <p>Dual-color FISH with differentially-labelled DNA probes for chromosomes 13 and 21</p>	<p>Centromeric signal was present in the majority of MN in Alzheimer's disease lymphocytes; aneuploidy for chromosome 21 was more frequent than for chromosome 13 in Alzheimer's disease patients</p> <p>FISH data showed that the percentage of centromere-negative MN was higher than that of centromere-positive MN in Parkinson's lymphocytes, which supports the theory that MN in Parkinson's originate from chromosomal breakage</p>

Key: Micronuclei (MN) containing centromere signal (C+ MN).

Table 4. Summary of Previous Reports Denoting the Presence of Centromeric and/or Telomeric Chromatin in MN After *in vitro* Therapeutic Treatments or Exposure to Hazardous Conditions.

Citation	Tissue	Study Sample	Technique	Key Findings
Digue et al. (1999) [94]	Human Lymphocytes	Paclitaxel-treated cells <i>in vitro</i>	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	>70% of C+ MN in treated cells contained two or more centromere signals >85% of paclitaxel-induced cells contained centromeric signal(s)
Thierens et al. (2000) [95]	Human Lymphocytes	Hospital workers occupationally exposed to radiation and a control group	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	There was a significant increase in centromere-positive MN for the radiation workers, while no effect on centromere-negative MN was present The observed systematic increase in micronucleus frequency with age was mainly due to increased chromosome loss, reflected in the centromere-positivity of the MN
Andrianopoulos et al. (2006) [96]	Human Lymphocytes	Hydrochlorothiazide-HCTZ-treated cells <i>in vitro</i>	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	Prevalence of Cx+ MN was significantly higher in cultures treated with higher concentrations of hydrochlorothiazide-HCTZ
Orsière et al. (2006) [97]	Human Lymphocytes	Peripheral blood samples obtained from pathologists/anatomists and a control group	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	The frequency of C+ MN was increased in pathologists/anatomists compared to controls: 78% of MN in pathologists/anatomists were C+ MN versus 67% of MN in controls
Iarmarcovai et al. (2006) [98]	Human Lymphocytes	Peripheral blood samples obtained from welders and a control group	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	Welders displayed an increased frequency of Cx + MN when compared to controls
Iarmarcovai et al. (2007) [99]	Human Lymphocytes	Pooled data, evaluating the effect of lifestyle factors - Blood samples obtained from cancer patients, pathologists/anatomists, and welders	Fluorescence <i>in situ</i> hybridization using a pancentromeric DNA probe	No effect of cigarette smoking was found Alcohol consumption had a significant effect on total MN frequency and particularly on centromere-positive MN

Key: Micronuclei (MN) containing centromere signal (C+ MN); MN specifically observed to contain two or more centromeres (Cx + MN).

4.2 Inflammation as a Potential Driver of Chromosomal Instability

Childhood adversity has diverse effects on physiological functioning across multiple axes: neural, endocrine, immune, metabolic, and gut microbial [40]. The impact of early-life stressful events include changes in brain structure and activity; neuroendocrine (hypothalamic-pituitary-adrenal [HPA]) stress response hyper- or hypo-reactivity generating excess “allostatic load;” parasympathetic- or sympathetic-predominant autonomic imbalances; chronic inflammation and immunosuppression; altered or impaired metabolism; and microbiome alterations [40]. Of particular relevance to the current study is the cascade of events that occur as a result of changes in physiology to the HPA stress response axes, as well as the immune and inflammatory systems. Various models proposed to explain how stress experienced in childhood may be biologically “remembered” to have lasting pathological consequences on the affected individual suggest that key players of the immune system become endowed with a proinflammatory phenotype, causing them to mount excessive inflammatory responses and become insensitive to inhibitory hormonal signals [40-41]. The result is a systemic state of mild inflammation in the body, and because the exposure to stress occurs in a period of development during which the immune system displays marked plasticity and is particularly impressionable to environmental insults, the proinflammatory phenotype becomes embedded and is maintained into adulthood [40-41]. This conjecture has been supported by the observation that adolescents and adults with a history of exposure to adverse early-life experiences have heightened immune responses to pathogenic stimuli and display elevated levels of important biomarkers of chronic inflammation (C-reactive protein [CRP], tumor necrosis factor-alpha [TNF- α], and the cytokine interleukin-6 [IL-6]) [40-41].

Inflammation is the initial immunologic response to tissue damage and pathogenic exposure that results in the delivery of white blood cells to the site of injury or infection [45]. Although critical for healing of wounds and for elimination of invading pathogens, the duration and extent of the inflammatory state must be carefully regulated [40-48]. When the inflammatory

stimulus persists, or when the immune response becomes deregulated, a state of chronic inflammation results, which may lead to a number of secondary consequences associated with heightened vulnerability to age-related health conditions [40-41,45]. Indeed, inflammation is a recognized feature underlying many chronic diseases of aging, such as atherosclerosis, arthritis, cancer, diabetes, osteoporosis, dementia, vascular diseases, autoimmune disorders, obesity, and metabolic syndrome [40-48].

One mechanism that has been proposed to be responsible for the programming of proinflammatory tendencies into cells is through epigenetic modifications to DNA [41]. Although investigators have yet to reach a unitary definition of epigenetics, the term has been used to describe any stable and mitotically (as well as meiotically) heritable change that occurs in the absence of a modification to the DNA sequence itself [70]. Epigenetic alterations, such as DNA methylation, are capable of altering transcriptional activity across thousands of genes and dozens of biological pathways – and can do so differently in identical twins [70]. Of particular interest to this proposal is the fact that epigenetics provides a means by which environmental factors can influence gene expression [70-71]. The fact that epigenetic alterations can be maintained from one somatic cell generation to the next has allowed the epigenetic model of embedding to become a particularly attractive means for explaining how psychological stress experienced in childhood could “incubate” in the body for many years before manifesting in disease [70].

Although chronic inflammation is thought to drive forward various mechanisms of pathogenesis – including high blood pressure, insulin resistance, plaque growth, tissue destruction, and tumor progression – the exact biological mechanisms linking the onset and progression of health problems with chronic inflammation have remained relatively enigmatic. However, a well-documented consequence of inflammation is chromosomal instability [49]. In light of the results from this study and previous studies in our laboratory, we propose that the inflammatory tendencies brought on by exposure to psychological stress in early life may, in

part, be driving the vulnerability to increased morbidity and mortality in adulthood by instigating chromosomal instability and the acquisition of chromosomal aberrations (Figure 5). Specifically, we propose that the state of chronic inflammation exhibited by individuals with a history of childhood adversity will foster an environment of oxidative and nitrosative stress – giving rise to a net excess of reactive chemical species – and promote telomere attrition, thereby pre-saging the development of chromosomal instability and the acquisition of chromosomal aberrations. Lastly, we acknowledge that, in addition to being a means for explaining how cells could become programmed with a proinflammatory phenotype, epigenetic alterations may also promote chromosomal instability.

Reactive chemical species (including superoxide [O₂], nitric oxide [NO], hydrogen peroxide [H₂O₂], and other reactive oxygen species [ROS], reactive nitrogen species [RNS], and reactive lipid species) are produced by macrophages and neutrophils (phagocytes) through the respiratory burst associated with the normal inflammatory processes; alternatively, reactive species may be induced by inflammatory cytokines such as TNF- α and IL-6 [45-49]. Through toxic interactions with nucleic acids, excess ROS and RNS can cause DNA damage by giving rise to single- and double- strand breaks, oxidized bases, abasic sites, DNA-DNA intrastrand adducts, and DNA-protein cross-links [45,51-52,72]. Yan et al. [72] recently described the genotoxic effects of inflammation-associated reactive species by reporting increased rates of gene mutations, gene amplification, micronucleus formation, and chromosomal instability observed in cells treated with TNF- α – all of which were effects that could be lessened by antioxidant supplementation [72]. Reactive species may also indirectly result in DNA damage by causing defects in DNA repair genes [45,73]. Furthermore, another well-established consequence of oxidative stress is telomere attrition [74].

Telomeres are specialized structures composed of nucleoprotein complexes that “cap” the distal ends of eukaryotic chromosomes [75]. Functional telomeres serve to shield DNA against degradation and to prevent illegitimate chromosome fusions [75-76]. Thus, telomeres

are required to protect chromosome stability, to maintain genomic integrity, and to ensure faithful segregation of genetic material into daughter cells at mitosis. In most somatic cell types, activity of the enzyme telomerase is insufficient to maintain telomere length, resulting in progressive telomere shortening with each round of DNA replication and cell division [77-78]. Given its high guanine content, telomeric DNA is also particularly susceptible to damage by reactive oxygen species [74]. The resultant dicentric chromosome is highly unstable and can lead to the creation of an anaphase bridge at mitosis, which then may be followed by breakage along the fused chromosomes – a phenomenon known as breakage-fusion-bridge (BFB) cycles. BFB cycles can end in the formation of chromosomal translocations, deletions, and amplifications [76]. In addition to structural aberrations, numerical abnormalities (i.e., aneuploidy) have been shown to result from telomere dysfunction and have been found to be associated with telomere attrition [76,79-80]. Although the mechanisms by which compromised telomere function or length may result in aneuploidy and are not yet clear, investigators have postulated that telomere dysfunction and/or shortening may: (i) compromise sister-telomere cohesion during mitosis; (ii) give rise to chromosome entanglement or misalignment due to illegitimate chromosome recombination or rearrangements; (iii) alter the expression of genes responsible for ensuring faithful mitotic processes; (iv) modify replication timing, leading to misalignment; (v) destabilize mitotic spindle attachment during mitosis; (vi) cause telomeric aggregates to form, leading to an uneven chromosome distribution during mitosis and subsequent loss of a chromosome in one daughter cell and gain of a chromosome in the other; and (vii) result in the development of end-to-end chromosome fusions that could segregate erroneously between daughter cells if microtubule attachments collapse at the kinetochores [65,76-80,105-106].

The effect of epigenetic changes on chromosomal stability has been observed in individuals with Immunodeficiency-Centromeric Instability-Facial Anomalies (ICF) syndrome, a condition caused by a mutation in the methyltransferases gene B that results in under-

condensation of the heterochromatic regions of chromosomes 1, 9, and 16 [81]. Individuals with this autosomal recessive disorder exhibit accelerated telomere attrition and have increased frequencies of acquired chromosomal aberrations; specifically, as a result of a loss of chromatin from chromosomes 1, 9, and 16 into MN [81]. There is also evidence to suggest that epigenetic modifications, especially those affecting the expression of histone or DNA methyltransferases, may have an impact on telomere length and, as discussed above, telomere dysfunction and telomere attrition are both avenues through which chromosomal instability may be achieved [82]. Furthermore, epigenetic alterations that result in hypermethylation of CpG islands within or adjacent to the promoter regions of housekeeping genes involved in cell-cycle checkpoints and DNA repair may increase the likelihood of malsegregation [81-82].

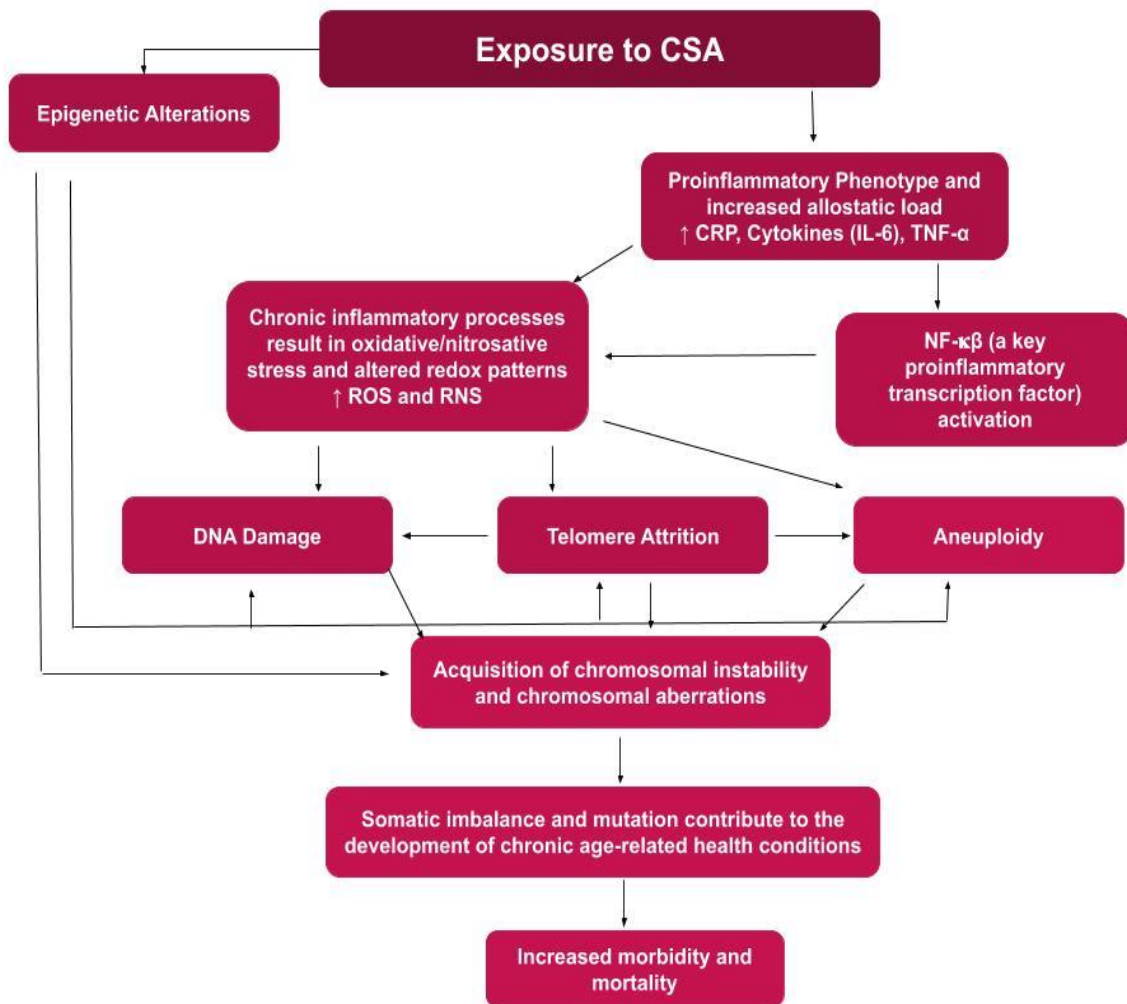


Figure 7. Proposed Chromosomal, Genetic, Epigenetic, and Cellular Attributes

Contributing to Somatic Cell Chromosomal Instability. Key: C-Reactive Protein (CRP);

interleukin-6 (IL-6); Tumor Necrosis Factor alpha (TNF-α); Reactive oxygen species (ROS);

Reactive nitrogen species (RNS) Chromosomal instability is broadly defined as the loss or gain of whole chromosomes (aneuploidy) and/or the acquisition of structural abnormalities.

4.3 Childhood Sexual Abuse and the Development of a Premature Aging Phenotype

The findings of the current study contribute to the bigger picture in providing evidence for a potential mechanism(s) based on increased chromosomal instability and the acquisition of chromosomal aberrations that could associate early-life stress and susceptibility to chronic age-related health conditions. Based on the knowledge that the incidence of acquired chromosomal abnormalities increases with advancing age, an estimated “biological age” of the adult female identical twins discordant for exposure to CSA was calculated [21]. These projections have suggested that, on the basis of frequencies of acquired chromosomal changes alone, a female identical twin exposed to CSA has a “biological age” that is, on average, 9.9 years older than her unexposed co-twin [21]. This finding, in conjunction with the observation that exposure to childhood adversity is a strong predictor of vulnerability to many chronic age-related health conditions and to early death in adulthood, has led our research team (and others) to conjecture that childhood adversity may contribute to premature aging cascade or spectrum [50].

In light of the conjectured premature aging phenotype attributed to individuals with a history of sexual abuse in childhood, it is important to point out that chronic inflammation, immune system dysregulation, redox imbalances caused by persistent oxidative stress and weakened antioxidant systems, and telomere attrition/dysfunction have all been associated with the aging process – and are all findings observed in individuals exposed to psychological stress in childhood [48,51-53]. In addition, chromosomal instability has been recognized as an underlying mechanism of human aging, with the resultant gene expression alterations being proposed to drive pathogenesis of the chronic age-related health conditions [54].

As mentioned above, telomere attrition is a well-documented consequence of the normal aging process [75]; however, accelerated telomere shortening may occur under conditions of chronic inflammation and oxidative and nitrosative stress [74]. One group of investigators evaluated the impact of childhood adversity on telomere length later in adulthood [20]. Not surprisingly, their results revealed that individuals reporting the experience of multiple childhood

adversities had significantly shorter telomeres than their peers [20]. Most strikingly, it was found that the difference in telomere length in individuals exposed to adversity in childhood, compared to individuals whose childhood was absent adversity, could translate into a 7- to 15-year difference in life span [20]. Although telomere lengths were not available for the full population of CSA-exposed twins studied here, using a chromosome-specific assay, we did observe significant telomere shortening in CSA+ twins (as compared to their co-twins) for a subset of these twins in a previous study [one could predict that heightened telomere shortening might be occurring in this group of women exposed to childhood trauma. We also found that familial support following a CSA incident can influence telomere length in adulthood, having a “resilience” effect [108].

Given the strong association between telomere attrition and chromosomal instability, it also seems plausible to suggest that shortened telomeres may influence the mechanism(s) leading to the formation of MN. As described earlier, both structural and numerical chromosomal abnormalities have been associated with telomere dysfunction and/or attrition [75-80,101-106]. With a diverse array of potential mechanisms proposed to explain how compromised telomere function or length may lead to aneuploidy, telomere shortening may offer one possible explanation for the increased frequency of MN arising from numerical errors in women exposed to CSA - a population likely to have experienced heightened telomere attrition in their cells. As an example, one could envisage that shortened telomeres may alter sister-telomere cohesion and alignment during mitosis, causing one or both chromatid(s) to lag behind at anaphase, and ultimately result in an aneuploid event and the formation of a micronucleus.

In light of the findings from the present study, it is also of interest to highlight the observation that metaphase spreads of cells that are nearing senescence due to critically short telomeres lacked a telomeric signal (i.e., chromatids displayed signal-free ends) using FISH with probes specific for the (TTAGGG)_n repeat sequence found in all human telomeres [104]. Indeed, in their analyses, 85% of metaphase spreads from near-senescent cell cultures displayed a

“signal-free end,” establishing that cells harboring telomeres sufficiently short to fail to produce a hybridization signal are still capable of undergoing cell division [104]. These investigators also showed that the shortest telomeres co-localized with γ H2AX/53BP1, which is indicative of DNA damage foci [104]. Thus, it seems not only possible - but likely - that chromosome(s) or chromatid(s) with critically short telomeres, or chromosomal fragments containing critically short telomeric regions would be inducted into MN, with the resultant MN being absent of a telomeric signal. Results from the present study indicated that women exposed to CSA had lower frequencies of MN containing terminal fragments (as indicated by the presence of a telomeric signal [but not a centromeric signal] within the micronucleus). Should women exposed to CSA have progressed to the point of harboring near-senescent cells due to significant telomere attrition in at least one or more chromosome(s), the observation that critically short telomeres may fail to display a hybridization signal offers an attractive and plausible explanation for our findings - and would provide further evidence in support of the conjectured “premature aging phenotype” exhibited by women exposed to CSA.

4.4 Methodological Strengths and Limitations

The present study comes on the heels of a landmark study by our laboratory identifying a direct link between CSA exposure and MN formation that is not attributable to genetic, environmental, or familial factors shared by siblings. A significant and important strength of this study and the previous study was the powerful model of the discordant identical (MZ) twin design, which allowed for the ability to control for potential confounding factors (such as familial and environmental influences outside of the exposure to CSA) as well as genetic contributions to enhance our ability to detect potential causality. We applied the discordant co-twin design to a novel and evolving approach for evaluating the contents of MN in an effort to better understand the biological and molecular impact of the increased frequency of MN formation. As stated earlier, the present study is the first time in which the combined FISH and CBMN assay

technique has been applied to better understand the type(s) of MN formed in relation to a powerful psychosocial factor: stress related to early adversity. Although relatively sparse, previous studies contributing to the body of knowledge examining the contents of MN have focused on understanding the type(s) of MN formed as a result of treatment to cells in culture, or in healthy adults and individuals living with a specific disease. The novelty of this study points to its innovation and originality.

Despite these strengths, there were methodological limitations to the study design that should be acknowledged. In particular, our studies were performed using lymphocytes, which is the cell that is typically used to evaluate chromosomes following stimulation with the mitogen phytohemagglutinin. Our observations of increased chromosomal or chromatid loss in cells of the immune system may therefore be most relevant to diseases and health conditions relating to the immune response and inflammatory system, but may or may not be appropriate for explaining the development of psychopathologies and mental health issues. However, it should be noted that chromosomal changes, especially aneuploidy, have a tendency to be acquired in multiple tissue types [54, 56-59]. Due to the ease and accessibility of studying lymphocytes, the vast majority of MN studies have been completed using this cell type and have indeed identified significantly increased frequencies of chromosomal abnormalities in individuals with a wide range of health problems (including psychopathologies) in lymphocytes [27-29].

Another weakness in our study design was the high percentage of CSA+ twins selected for study, rather than collecting data points on an equal number of CSA- twins as well as healthy, aging twins for our reference control group. In particular, despite the, powerful discordant twin pair design model of the study, the small number of complete discordant twin pairs studied here may have made it difficult to detect moderate effects on the number of MN arising through a numerical versus structural event among CSA+ and CSA- co-twins.

Other limitations of the current study include the high variation in MN counts for each of the categories assessed, as indicated by the large standard deviations observed. As this is still

an emerging area of study, we do not yet know if there may be other factors impacting the types of MN observed here.

4.5 Future Directions

Although the methodology used in the present study is informative for differentiating between MN harboring intact versus fragmented chromosomes, this technique does not have the capacity to unveil a complete picture of the mechanisms underlying MN formation. More specifically, the application of pancentromeric and pantelomeric FISH probes on cytokinesis-blocked lymphocytes did not evaluate whether *specific* chromosomes are preferentially excluded into MN as either whole, intact chromosomes/chromatids or as chromosomal fragments. SKY or the use of FISH with probes specific for the pericentromeric regions of distinct chromosomes are two techniques that could be used to assess exclusion of *specific* chromosomes into MN. These methods could also provide data regarding whether the chromosomal distribution in MN differs based on exposure to CSA. Indeed, preliminary SKY analysis using participants from the same population of participants studied here showed that the majority (89.3%) of MN contained chromatin from a single chromosome, and chromatin from chromosomes X (23%; $\chi^2_{23}=7122$; $P<0.001$) and 8 (8%; $\chi^2_{23}=31.51$; $P<0.001$) were seen more frequently than expected by chance [60].

Additionally, in light of the conjecture that the mechanism(s) leading to MN formation may differ for MN containing only one centromere compared to MN containing more than one centromere [93], it may be informative to evaluate the number of MN arising from chromatid- versus chromosome-type errors in our study population. This type of analysis could be achieved by assessing the number of centromeric (and potentially also telomeric) signals present in MN.

Another avenue for future study is to consider whether the exclusion of chromosomes/chromatids or chromosomal fragments into MN may be occurring as either a “corrective” or an “error”-based mechanism. While the formation of MN is usually considered to

result in a somatic imbalance in one or both of the daughter binucleates, recent studies by our research team have suggested that MN may arise through a “corrective” or protective mechanism, whereby DNA imbalances are eliminated from the parent cell in an attempt to restore the normal, euploid chromosomal complement at the completion of the cell cycle [22]. One could envisage this “correction” process playing out in either a trisomy rescue mechanism or as a defense mechanism against the formation of double minutes [22]. To better understand whether the exclusion of chromosomes/chromatids or chromosomal fragments into MN may be occurring as either a “corrective” or an “error”-based mechanism (and whether these mechanisms differ based on exposure to CSA), the number of signals present in MN and their corresponding binucleates could be scored in cytokinesis-blocked cultured lymphocytes following FISH with probes specific for the pericentromeric regions of distinct chromosomes.

Finally, it remains to be seen whether the acquired chromosomal aberrations observed here (and previously) may result in clonal expansion of cells with an abnormal karyotype and subsequent development of somatic mosaicism – a recently identified phenomenon that has also been strongly associated with advancing age [61-63]. Mosaicism develops when a genetic alteration – including single nucleotide substitutions, copy number variants (CNVs), copy-neutral loss of heterozygosity, chromosome rearrangements, and aneuploidy – is propagated to a small subset of somatic cells [64]. In recent studies, investigators have demonstrated that somatic mosaicism of chromosomal abnormalities may, in fact, be more prevalent than previously anticipated, with rates of chromosomal mosaicism in adult brain cells potentially reaching 10% [64]. While the consequences of somatic mosaicism are dependent on the underlying altered genetic architecture, investigators now speculate that chromosomal mosaicism “may play a relevant role in human diversity and disease susceptibility” - and may help to explain the biological basis for the increased morbidity and mortality in individuals who experienced abuse in childhood [65-66]. Indeed, somatic mosaicism has recently been recognized in the pathogenesis of many human diseases – both inherited and acquired – with mosaicism for

genetic abnormalities playing a critical role in the onset and progression of cancer and being an established cause of miscarriage, infertility, birth defects, and developmental delay [61-67]. In addition, investigators have suggested that somatic mosaicism may, in part, be responsible for the development of many complex disorders such as autism, schizophrenia, autoimmune conditions, and Alzheimer's disease, with an age-related increase in the prevalence of mosaic anomalies likely being an underlying cause of disease onset for many of these conditions [54]. Furthermore, somatic mosaicism has recently been recognized to contribute to the divergence of phenotypes of identical twins, whose genomes are indistinguishable at conception [54,61,66].

Detectable clonal mosaicism describes the observation of a sub-population of cells, presumably of clonal origin, with the same abnormal karyotype that is present at sufficient frequency (>5-10%) within a population of normal cells to be detected using SNP microarray or other DNA-based methods [61-63]. In two landmark studies performed by Jacobs et al. [62] and Laurie et al. [63], the presence of detectable clonal mosaicism was found to be associated with both advancing age and cancer. Hypothetically, the presence of stress-related oxidative damage and accelerated telomere shortening could exacerbate the age-related reduction in the diversity of cell clones [61], increasing the probability that an aberrant cell would propagate and be detected as clonal mosaicism – and again further increasing the propensity of that abnormal karyotype to have an impact on the overall phenotype of the individual. Alternatively, the increased frequency of clonal mosaic anomalies detected in older adults could be a consequence of the increased rates of chromosomal instability in the elderly (and in our population of adult female identical twins exposed to CSA) [22,29]. Given the relationship between exposure to CSA and the tendency to acquire chromosomal changes that result in elevated MN frequencies, it seems plausible to predict that adult female identical twins with a history of CSA are more likely to develop an aberration that has the potential to clonally expand, giving rise to detectable clonal mosaicism, than their unexposed co-twins.

4.6 Summary

In summary, we identified intact chromosome/chromatid loss during the mitotic process as a mechanism potentially underlying the increased frequencies of MN present in peripheral blood lymphocytes of adult female identical twins with a history of exposure to childhood sexual abuse. MN harboring fragmented chromosomes were also observed in all of the study participants evaluated. Given that aneuploidy and chromosomal breakage contributes to the development of age-related diseases, these observations are an important step toward understanding the latent biological consequences of childhood adversity on future health outcomes. Further understanding of the biological impact of stressful early-life events could provide important foundational knowledge for ultimately creating screening tools to identify individuals “at risk” for developing stress-associated and age-related health conditions in adulthood.

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Appendix: R Output

1.1 Comparison of Overall MN Frequencies (Pairwise Comparisons) Within Discordant Twin Pairs

```
#Read Data
```

```
ref <- read.csv("AllTwinsReference.csv", header = T, na.strings = "")
```

```
#Set-up
```

```
install.packages("reshape2")
```

```
library('reshape2')
```

```
install.packages("arsenal")
```

```
library("arsenal")
```

```
install.packages("dplyr")
```

```
library("dplyr")
```

```
install.packages("PairedData")
```

```
library("PairedData")
```

```
> ##Compare MN Frequencies Within Discordant Twin Pairs
```

```
>
```

```
> exposed <- c(15.5,
```

```
+      12.5,
```

```
+      7.5,
```

```
+      6.5,
```

```
+      23,
```

```
+      18,
```

```

+      11.5,
+      33,
+      7.5,
+      29.5,
+      18.5,
+      33.5,
+      30,
+      45.5,
+      30.5,
+      30)
>
> unexposed <- c(11.5,
+      23,
+      8.5,
+      14,
+      19,
+      16,
+      12,
+      18.5,
+      2.5,
+      12.5,
+      9.5,
+      22.5,
+      13.5,
+      11.5,
+      17,

```

```

+           19.5)
>
> my_paired_data <- data.frame(
+   group = rep(c("exposed", "unexposed"), each=16),
+   whole = c(exposed, unexposed)
+ )
> exposed <- subset(my_paired_data, group == "exposed", whole,
+   drop=TRUE)
> unexposed <- subset(my_paired_data, group == "unexposed", whole,
+   drop=TRUE)
> pd <- paired(exposed, unexposed)
> plot(pd, type = "profile") + theme_bw()
> res <- wilcox.test(exposed, unexposed, paired = TRUE)

```

Warning message:

In wilcox.test.default(exposed, unexposed, paired = TRUE) :

cannot compute exact p-value with ties

```
> res
```

Wilcoxon signed rank test with continuity correction

data: exposed and unexposed

$V = 116.5$, $p\text{-value} = 0.01303$

alternative hypothesis: true location shift is not equal to 0

```

> d <- with(my_paired_data, whole[group == "exposed"] - whole[group == "unexposed"])
> d

```

```
[1] 4.0 -10.5 -1.0 -7.5 4.0 2.0 -0.5 14.5 5.0 17.0 9.0 11.0 16.5 34.0 13.5 10.5
```

```
> shapiro.test(d)
```

Shapiro-Wilk normality test

data: d

W = 0.9592, p-value = 0.6474

```
> res1 <- t.test(exposed, unexposed, paired = TRUE)
```

```
> res1
```

Paired t-test

data: exposed and unexposed

t = 2.8299, df = 15, p-value = 0.01267

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

1.87429 13.31321

sample estimates:

mean of the differences

7.59375

1.2 Comparison of Overall MN Frequencies Between CSA+ and CSA- Twin Groups (Group Comparisons)

```
t.test(ref[ref$csastatus=="Exposed",  
"mnfrequency"],ref[ref$csastatus=="Unexposed","mnfrequency"])
```

Welch Two Sample t-test

data: ref[ref\$csastatus == "Exposed", "mnfrequency"] and ref[ref\$csastatus == "Unexposed",
"mnfrequency"]

t = 2.51, df = 50.023, p-value = 0.01536

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

1.18167 10.64847

sample estimates:

mean of x mean of y

22.46053 16.54545

1.3 Linear Mixed-Effect Models Evaluating Overall MN Frequencies

```
# SETUP -----
```

```
library('tidyverse')
```

```
#install.packages('lme4')
```

```
library('lme4')    #for linear mixed effect models
```

```
#install.packages('lmerTest')
```

```
library('lmerTest')
```

```
#install.packages('skimr')
```

```
library('skimr')
```

```

# READ DATA -----
ref <- as_tibble(read.csv("AllTwinsReference.csv", header = T, na.strings = ""))

#MN Frequency Linear Mixed-Effects Modeling

> temp5 <- ref %>%
+   select(csastatus, exposurestatus, tidno, age, mnfrequency) %>%
+   mutate(csa= as.factor(ifelse(csastatus=="Exposed", 1, 0))) %>%
+   mutate(mnf = sqrt(mnfrequency)) %>%
+   mutate(fam.exp= as.factor(ifelse(csastatus=="Reference", 0, 1))) %>%
+   mutate(fid= (tidno)) %>%
+   select(fid, csa, age, mnf, fam.exp)
>
>
> #add missing family ids
> fid2 <- 1
> for (i in 1:length(temp5$fid)) {
+   if (is.na(temp5$fid[i])) {
+     temp5$fid[i] <- fid2
+     fid2 <- fid2 + 1
+   }
+ }
> table(is.na(temp5$fid))

FALSE

```



```

>
> # MODEL FITTING -----
>
> # csa only
> out <- lmer(mnf ~ csa + (1|fid), data= temp5)      #significant
> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: mnf ~ csa + (1 | fid)

Data: temp5

REML criterion at convergence: 189.5

Scaled residuals:

    Min     1Q  Median     3Q     Max
-1.79191 -0.63192  0.02774  0.60229  1.99124

Random effects:

Groups   Name      Variance Std.Dev.
fid      (Intercept) 0.3787   0.6154
Residual                0.6749   0.8215

Number of obs: 66, groups: fid, 48

Fixed effects:

            Estimate Std. Error   df t value Pr(>|t|)
(Intercept)  4.1546    0.1918 63.9557  21.661  <2e-16 ***
csa1         0.5445    0.2344 41.6931   2.323  0.0251 *

```

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr)

csa1 -0.702

>

> # age only

> out <- lmer(mnf ~ age + (1|fid), data= temp5) #not significant

> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: mnf ~ age + (1 | fid)

Data: temp5

REML criterion at convergence: 198.2

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.18444	-0.66162	-0.02481	0.67741	1.94201

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	0.2078	0.4558
	Residual	0.8643	0.9297

Number of obs: 66, groups: fid, 48

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	3.53483	0.71342	45.20456	4.955	1.06e-05 ***
age	0.01762	0.01353	46.34999	1.303	0.199

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

```
(Intr)
age -0.982
>
>
> # fam.exp only
> out <- lmer(mnf ~ fam.exp + (1|fid), data= temp5)    #not significant
> summary(out)
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: mnf ~ fam.exp + (1 | fid)
Data: temp5
```

REML criterion at convergence: 191.8

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.30784	-0.65017	-0.07103	0.65330	2.07038

Random effects:

Groups Name Variance Std.Dev.

fid (Intercept) 0.1740 0.4171

Residual 0.9043 0.9510

Number of obs: 66, groups: fid, 48

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	4.8636	0.4239	62.5642	11.473	<2e-16 ***
fam.exp1	-0.4659	0.4465	60.9618	-1.044	0.301

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr)

fam.exp1 -0.950

>

> # csa and fam.exp

> out <- lmer(mnf ~ csa + fam.exp + (1|fid), data= temp5) #csa still significant

> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: mnf ~ csa + fam.exp + (1 | fid)

Data: temp5

REML criterion at convergence: 185.4

Scaled residuals:

Min	1Q	Median	3Q	Max
-1.87115	-0.52390	-0.02103	0.60967	2.21181

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	0.3472	0.5893
Residual		0.6592	0.8119

Number of obs: 66, groups: fid, 48

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	4.8636	0.4095	57.9485	11.876	< 2e-16 ***
csa1	0.7038	0.2451	37.3339	2.872	0.00669 **
fam.exp1	-0.8971	0.4608	59.9580	-1.947	0.05626 .

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	csa1
csa1	0.000	
fam.exp1	-0.889	-0.342

2.1 Comparing the Frequencies of MN Harboring Intact Chromosomes or Chromatids in CSA+ and CSA- Twin Groups

```
> wilcox.test(ref[ref$csastatus == "Unexposed", "whole"], ref[ref$csastatus == "Exposed",
"whole"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Unexposed", "whole"] and ref[ref\$csastatus == "Exposed", "whole"]

W = 456.5, p-value = 0.01415

alternative hypothesis: true location shift is not equal to 0

```
> wilcox.test(ref[ref$csastatus == "Exposed", "whole"], ref[ref$csastatus == "Reference",  
"whole"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Exposed", "whole"] and ref[ref\$csastatus == "Reference", "whole"]

W = 303, p-value = 0.008657

alternative hypothesis: true location shift is not equal to 0

```
> wilcox.test(ref[ref$csastatus == "Unexposed", "whole"], ref[ref$csastatus == "Reference",  
"whole"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Unexposed", "whole"] and ref[ref\$csastatus == "Reference",
"whole"]

W = 98, p-value = 0.1245

alternative hypothesis: true location shift is not equal to 0

```
In wilcox.test.default(ref[ref$csastatus == "Unexposed", "whole"], :
cannot compute exact p-value with ties
```

```
# SETUP -----

library('tidyverse')

#install.packages('lme4')

library('lme4')    #for linear mixed effect models

#install.packages('lmerTest')

library('lmerTest')

#install.packages('skimr')

library('skimr')


# READ DATA -----

ref <- as_tibble(read.csv("AllTwinsReference.csv", header = T, na.strings = ""))

> #specify simple tibble

> temp <- ref %>%

+   select(whole, csastatus, exposurestatus, tidno, age) %>%

+   mutate(csa= as.factor(ifelse(csastatus=="Exposed", 1, 0))) %>%

+   mutate(wholefreq = sqrt(whole)) %>%

+   mutate(fam.exp= as.factor(ifelse(csastatus=="Reference", 0, 1))) %>%

+   mutate(fid= (tidno)) %>%

+   select(fid, csa, wholefreq, age, fam.exp)

>
```

```

>
> #add missing family ids
> fid2 <- 1
> for (i in 1:length(temp$fid)) {
+   if (is.na(temp$fid[i])) {
+     temp$fid[i] <- fid2
+     fid2 <- fid2 + 1
+   }
+ }
> table(is.na(temp$fid))

```

FALSE

90

```

>

```

```

>

```

```

> # MODEL FITTING -----

```

```

>

```

```

> # age only

```

```

> out <- lmer(wholefreq ~ age + (1|fid), data= temp)      #not significant

```

```

> summary(out)

```

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: wholefreq ~ age + (1 | fid)

Data: temp

REML criterion at convergence: 307.1

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.12008	-0.34580	0.09719	0.48196	1.62417

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	1.3527	1.1630
	Residual	0.6262	0.7913

Number of obs: 90, groups: fid, 65

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	8.099063	0.929339	68.402071	8.715	1.06e-12 ***
age	-0.006271	0.017448	68.725043	-0.359	0.72

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

```
(Intr)
age -0.983
>
>
> # fam.exp only
> out <- lmer(wholefreq ~ fam.exp + (1|fid), data= temp)    #not significant
> summary(out)
```

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: wholefreq ~ fam.exp + (1 | fid)

Data: temp

REML criterion at convergence: 291.8

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.1748	-0.3671	0.1166	0.4953	1.5565

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	1.1742	1.0836
	Residual	0.6015	0.7755

Number of obs: 90, groups: fid, 65

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	6.2303	0.5440	73.7001	11.45	< 2e-16 ***
fam.exp1	1.6840	0.5689	72.8311	2.96	0.00415 **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)
fam.exp1	-0.956

>

```

>
> # csa only
> out <- lmer(wholefreq ~ csa + (1|fid), data= temp)      #significant
> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: wholefreq ~ csa + (1 | fid)

Data: temp

REML criterion at convergence: 295.4

Scaled residuals:

    Min     1Q  Median     3Q     Max
-2.0361 -0.3320  0.1474  0.4546  1.5212

Random effects:

Groups   Name      Variance Std.Dev.
fid      (Intercept) 1.1155   1.0562
Residual                0.6607   0.8128

Number of obs: 90, groups: fid, 65

Fixed effects:

              Estimate Std. Error    df t value Pr(>|t|)
(Intercept)  7.3305     0.2295 87.1086  31.937  <2e-16 ***
csa1          0.6537     0.2472 54.6321   2.645  0.0106 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

Correlation of Fixed Effects:

(Intr)

csa1 -0.723

>

>

> # csa + age

> out <- lmer(wholefreq ~ csa + age + (1|fid), data= temp) #csa significant

> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: wholefreq ~ csa + age + (1 | fid)

Data: temp

REML criterion at convergence: 301.4

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.0517	-0.3373	0.1187	0.4649	1.4999

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	1.1196	1.0581
	Residual	0.6687	0.8177

Number of obs: 90, groups: fid, 65

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	7.807496	0.889433	68.146475	8.778	8.36e-13 ***
csa1	0.661629	0.248652	54.873371	2.661	0.0102 *
age	-0.009208	0.016550	65.873719	-0.556	0.5798

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr) csa1

csa1 -0.142

age -0.966 -0.047

>

>

> # csa + fam.exp

> out <- lmer(wholefreq ~ csa + fam.exp + (1|fid), data= temp) #csa borderline significant,

fam.exp significant

> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: wholefreq ~ csa + fam.exp + (1 | fid)

Data: temp

REML criterion at convergence: 289.5

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.0091	-0.3874	0.1398	0.4669	1.4062

Random effects:

Groups	Name	Variance	Std.Dev.
--------	------	----------	----------

fid	(Intercept)	1.0867	1.042
-----	-------------	--------	-------

Residual		0.6178	0.786
----------	--	--------	-------

Number of obs: 90, groups: fid, 65

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	6.2303	0.5330	73.2876	11.689	<2e-16 ***
csa1	0.4634	0.2533	49.5422	1.829	0.0734 .
fam.exp1	1.3424	0.5874	78.9210	2.285	0.0250 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	csa1
csa1		0.000
fam.exp1	-0.907	-0.317

>

>

> # csa + age + fam.exp

> out <- lmer(wholefreq ~ csa + age + fam.exp + (1|fid), data= temp) #csa borderline
significant, fam.exp remains significant, age not significant

> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: wholefreq ~ csa + age + fam.exp + (1 | fid)

Data: temp

REML criterion at convergence: 295.8

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.0085	-0.3638	0.1380	0.4612	1.3967

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	1.1028	1.0501
	Residual	0.6223	0.7889

Number of obs: 90, groups: fid, 65

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	6.436412	1.070682	68.312010	6.012	8.01e-08 ***
csa1	0.467771	0.255540	49.122999	1.831	0.0732 .
age	-0.003658	0.016451	67.034982	-0.222	0.8247
fam.exp1	1.323521	0.597470	77.740519	2.215	0.0297 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr) csa1 age

```
csa1    0.080
age     -0.866 -0.092
fam.exp1 -0.578 -0.325 0.148
```

```
> #Adding in Overall MN Frequency Assessment

> temp3 <- ref %>%

+ select(whole, csastatus, exposurestatus, tidno, age, mnfrequency) %>%

+ mutate(csa= as.factor(ifelse(csastatus=="Exposed", 1, 0))) %>%

+ mutate(wholefreq = sqrt(whole)) %>%

+ mutate(fam.exp= as.factor(ifelse(csastatus=="Reference", 0, 1))) %>%

+ mutate(fid= (tidno)) %>%

+ mutate(mn= (mnfrequency)) %>%

+ select(fid, csa, wholefreq, age, mn, fam.exp)

>

>

> #add missing family ids

> fid2 <- 1

> for (i in 1:length(temp3$fid)) {

+   if (is.na(temp3$fid[i])) {

+     temp3$fid[i] <- fid2

+     fid2 <- fid2 + 1

+   }

+ }

> table(is.na(temp3$fid))
```


FALSE

90

>

>

>

> # MODEL FITTING -----

>

> # overall mn frequency only

> out <- lmer(wholefreq ~ mn + (1|fid), data= temp3) #not significant

> summary(out)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: wholefreq ~ mn + (1 | fid)

Data: temp3

REML criterion at convergence: 216.1

Scaled residuals:

Min	1Q	Median	3Q	Max
-1.7626	-0.4421	0.1848	0.6023	1.4183

Random effects:

Groups	Name	Variance	Std.Dev.
fid	(Intercept)	0.5564	0.7459
Residual		0.9113	0.9546

Number of obs: 66, groups: fid, 48

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	7.00031	0.37077	63.27073	18.881	<2e-16 ***
mn	0.01028	0.01584	62.55248	0.649	0.519

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr)

mn -0.900

2.3 Correlation of CSA Exposure and Shared Family Environment

```
> library('polycor')  
> hetcor(as.data.frame(temp[, c('csa','age','fam.exp')]))
```

Two-Step Estimates

Correlations/Type of Correlation:

	csa	age	fam.exp
csa	1	Polyserial	Polychoric
age	0.07163	1	Polyserial
fam.exp	0.9752	-0.1511	1

Standard Errors:

	csa	age
csa		
age	0.1336	
fam.exp	544.9	0.1657

n = 90

P-values for Tests of Bivariate Normality:

```
      csa  age
csa
age  0.5961
fam.exp  <NA> 0.5315
```

Warning message:

```
In hetcor.data.frame(as.data.frame(temp[, c("csa", "age", "fam.exp")])) :
  the correlation matrix has been adjusted to make it positive-definite
> table(temp$csa, temp$fam.exp)
```

```
  0 1
0 6 23
1 0 61
> fisher.test(temp$csa, temp$fam.exp)
```

Fisher's Exact Test for Count Data

```
data: temp$csa and temp$fam.exp
p-value = 0.0007629
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 2.808881      Inf
sample estimates:
odds ratio
      Inf
```

2.4 Comparing the Frequencies of MN Arising from Structural Abnormalities in CSA+ and CSA- Twin Groups

```
> wilcox.test(ref[ref$scsastatus == "Exposed", "terminal"], ref[ref$scsastatus == "Unexposed",
"terminal"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Exposed", "terminal"] and ref[ref\$csastatus == "Unexposed",
"terminal"]

W = 481.5, p-value = 0.02761

alternative hypothesis: true location shift is not equal to 0

```
> wilcox.test(ref[ref$csastatus == "Exposed", "structural"], ref[ref$csastatus == "Unexposed",  
"structural"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Exposed", "structural"] and ref[ref\$csastatus == "Unexposed",
"structural"]

W = 456.5, p-value = 0.01415

alternative hypothesis: true location shift is not equal to 0

```
> wilcox.test(ref[ref$csastatus == "Exposed", "acentric"], ref[ref$csastatus == "Unexposed",  
"acentric"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Exposed", "acentric"] and ref[ref\$csastatus == "Unexposed",
"acentric"]

W = 495, p-value = 0.03748

alternative hypothesis: true location shift is not equal to 0

```
> wilcox.test(ref[ref$csastatus == "Exposed", "centric"], ref[ref$csastatus == "Unexposed",  
"centric"])
```

Wilcoxon rank sum test with continuity correction

data: ref[ref\$csastatus == "Exposed", "centric"] and ref[ref\$csastatus == "Unexposed",
"centric"]

W = 583.5, p-value = 0.2284

alternative hypothesis: true location shift is not equal to 0

2.5 Relationship Between CSA Class and Frequency of MN Arising from a Numerical Event

```
> summary(lm(whole ~ csaclass,data=ref))
```

Call:

```
lm(formula = whole ~ csaclass, data = ref)
```

Residuals:

Min	1Q	Median	3Q	Max
-41.790	-16.368	0.054	16.606	31.658

Coefficients:

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	61.446	3.732	16.463	<2e-16 ***
csaclass	1.448	2.002	0.723	0.472

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 20.32 on 82 degrees of freedom

(6 observations deleted due to missingness)

Multiple R-squared: 0.006339, Adjusted R-squared: -0.005779

F-statistic: 0.5231 on 1 and 82 DF, p-value: 0.4716

2.6 Pairwise Comparisons of Intact Chromosome-Derived MN Frequencies Within Discordant Twin Pairs

```
> #Pairwise Comparisons of Whole Chromosome-Derived MN Frequencies among Discordant Twin Pairs
```

```
> exposed <- c(57,
```

```
+      78,
```

```
+      47,
```

```
+      45,
```

```
+      54,
```

```

+      57,
+      55,
+      55,
+      46,
+      48,
+      35,
+      71,
+      24,
+      28,
+      29,
+      55)
>
> unexposed <- c(69,
+      62.1052632,
+      28,
+      36,
+      80,
+      51,
+      32,
+      24,
+      61,
+      44,
+      31.3131313,
+      44,
+      45,
+      73,
+      51,
+      60)
>
> my_paired_data <- data.frame(
+   group = rep(c("exposed", "unexposed"), each=16),
+   whole = c(exposed, unexposed))
>
>

```

```

> exposed <- subset(my_paired_data, group == "exposed", whole, drop=TRUE)
> unexposed <- subset(my_paired_data, group == "unexposed", whole, drop=TRUE)
> pd <- paired(exposed, unexposed)
> plot(pd, type = "profile") + theme_bw()
>
> res <- wilcox.test(exposed, unexposed, paired = TRUE)
> res

```

Wilcoxon signed rank test

data: exposed and unexposed

$V = 70$, p-value = 0.9399

alternative hypothesis: true location shift is not equal to 0

```

> d <- with(my_paired_data, whole[group == "exposed"] - whole[group == "unexposed"])
> d
[1] -12.000000 15.894737 19.000000 9.000000 -26.000000 6.000000 23.000000
31.000000 -15.000000
[10] 4.000000 3.686869 27.000000 -21.000000 -45.000000 -22.000000 -5.000000
> shapiro.test(d)

```

Shapiro-Wilk normality test

data: d

$W = 0.96594$, p-value = 0.7694

```

> res1 <- t.test(exposed, unexposed, paired = TRUE)
> res1

```

Paired t-test

data: exposed and unexposed

$t = -0.085877$, $df = 15$, p-value = 0.9327

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-11.97134 11.04404

sample estimates:

mean of the differences

-0.4636497

2.7 Pairwise Comparisons of Intact Chromosome-Derived MN Frequencies Within Concordant Twin Pairs

```
> #Compare Whole Chromosome-Derived MN Frequencies Within Concordant Twin Pairs
```

```
> twina <- c(62,72,55,35,96,78,90,90,91,81,77)
```

```
>
```

```
> twinb <- c(72,75,48,40,94,84,91,94,89,78,88)
```

```
>
```

```
> my_paired_data <- data.frame(
```

```
+ group = rep(c("twina", "twinb"), each=11),
```

```
+ whole = c(twina, twinb)
```

```
+ )
```

```
>
```

```
> twina <- subset(my_paired_data, group == "twina", whole,
```

```
+ drop=TRUE)
```

```
> twinb <- subset(my_paired_data, group == "twinb", whole,
```

```
+ drop=TRUE)
```

```
> pd <- paired(twina, twinb)
```

```
> plot(pd, type = "profile") + theme_bw()
```

```
> res <- wilcox.test(twina, twinb, paired = TRUE)
```

Warning message:

In wilcox.test.default(twina, twinb, paired = TRUE) :

cannot compute exact p-value with ties

```
> res
```

Wilcoxon signed rank test with continuity correction

data: twina and twinb

V = 18.5, p-value = 0.2128

alternative hypothesis: true location shift is not equal to 0

```
> d <- with(my_paired_data, whole[group == "twina"] - whole[group == "twinb"])
> d
[1] -10 -3 7 -5 2 -6 -1 -4 2 3 -11
> shapiro.test(d)
```

Shapiro-Wilk normality test

data: d

W = 0.96984, p-value = 0.885

```
> res1 <- t.test(twina, twinb, paired = TRUE)
> res1
```

Paired t-test

data: twina and twinb

t = -1.4022, df = 10, p-value = 0.1911

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-6.119437 1.392164

sample estimates:

mean of the differences

-2.363636

Vita

Kaitlyn Marie Dochelli (legal married name: Connelly) was born on November 11th, 1987 in Philadelphia, Pennsylvania. She graduated from Central Bucks High School West in Doylestown, Pennsylvania in 2006. She received her Bachelor of Arts degree in Biology from the University of Richmond, Richmond, Virginia in May 2010. She began her graduate studies in Human Genetics at Virginia Commonwealth University in August 2010, but stepped away from the program in August 2015. After working at LuMind Research Down Syndrome for 1.5 years (August 2015 - January 2017) and subsequently at the Alzheimer's Association for the last three years (January 2017 - present), Kaitlyn returned to Virginia Commonwealth University to complete her M.S. degree in Human Genetics. She is currently living in Denver, Colorado.